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(54) Title: INHIBITOR OF DIFFERENTIATION KNOCKOUT MAMMALS AND METHODS OF USE THEREOF

(57) Abstract: This invention relates to Id knockout mammals having a disruption in at least one and at most three alleles of inhibitor of differentiation genes, Id1 and Id3. This results in reduction or prevention of a cell proliferative disorder in the mammal as compared to a wild-type mammal. In particular, tumor growth and/or metastasis is shown to be inhibited. Further, tumor growth is shown to have poor vascularization and extensive necrosis in Id knockout mammals lacking 3 out of 4 of the Id1, Id3 alleles (Id1-/Id3+/-). Drug screening methods to select agents useful to affect activity or expression of Id1, or Id3 gene products are disclosed. Therapeutic methods employing selected agents in subjects in need of treatment and diagnostic methods and test kits to identify subjects having, or at risk of having a neurological or cell proliferative disorder are also described.

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INHIBITOR OF DIFFERENTIATION KNOCKOUT MAMMALS AND METHODS OF USE THEREOF

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FIELD OF THE INVENTION

The present invention relates to knockout mammals having a disruption in one or more inhibitor of differentiation genes, Id1 and Id3, resulting in reduction or prevention of a cell proliferative disorder in the mammal. In particular, this invention relates to methods of preventing, ameliorating, or treating diseases related to neurogenesis and cell proliferative disorders by agents that affect the activity and/or expression of one or more Id gene products. Drug screening methods to select these agents, and diagnostic methods, and test kits to identify whether a subject has, or is at risk of developing, a neurological or cell proliferative disorder also are described.

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BACKGROUND OF THE INVENTION

Inhibitor of differentiation (Id) genes encode members of the helix-loop-helix (HLH) family of transcription factors that inhibit transcription by forming inactive heterodimers with basic HLH (bHLH) proteins. There are four members of the Id gene family recognized in mammals, and the proteins they encode share homology primarily in their HLH domain. Typically, bHLH proteins form heterodimers with other bHLH proteins, and their basic domain binds to a DNA sequence element, the E-box, activating transcription. Products of Id genes lack the basic DNA binding domain of the bHLH transcription factors, and when they heterodimerize with bHLH proteins, the resultant complexes are inactive. Id proteins play a major role in cell growth and differentiation. Id proteins function at a general level as positive regulators of cell growth and as negative regulators of cell differentiation. The importance of Id proteins as regulatory intermediates for coordinating differentiation-linked gene expression has been documented. For a review see, for example, Norton *et al.*, *Trends Cell Biol.* 8(2):58-65(1998). Overexpression of Id blocks bHLH-mediated transcription in a wide variety of cell types. Generally, high levels of Id mRNA are detected in proliferative undifferentiated, embryonic cells, and lower levels are detected in well-differentiated, mature and adult tissues. *In vitro*, these genes are generally expressed at lower levels in

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cells after the induction of differentiation. Thus, it is recognized that Id proteins inhibit differentiation and enhance cell proliferation.

Recently, high levels of expression of Id genes have been identified in cell lines derived from a wide variety of different tumors. Because bHLH complexes regulated by Id may have varied effects on the differentiated state of a cell, overexpression or upregulation of Id has been associated with a block to differentiation (Deed *et al.*, *J. Biol. Chem.* 27:8278-8286 (1998); Ogata *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 9219-9222 (1993); Neuman *et al.*, *Dev. Biol.* 160:186-195 (1993)) or an alteration in cell fate (Heemskerk, *et al.*, *J. Exp. Med.* 186:1597-1602 (1997); Martinsen *et al.*, *Science* 281:988-991 (1998); Katagiri *et al.*, *J. Cell. Biol.* 127:1755-1766 (1994)) in different cell contexts.

Id gene expression has been found to be a key mediator of tumor cell biology. See, for example, Israel *et al.*, *Cancer Res.* 1 (59):1726-1730 (1999); and Maruyama *et al.*, *Am. J. Pathol.* 155(3):815-22(1999). High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double- knockout mice is shown to be associated with enhanced proliferative potential of pancreatic cancer cells and of proliferating or dysplastic ductal cells in chronic pancreatitis. Thus, not only the inappropriate proliferation of tumors but also the anaplastic characteristics that contribute to their malignant behavior may be regulated by Id gene expression.

Additionally, it has been shown that Id1 and Id3 are co-expressed temporally and spatially during murine neurogenesis and angiogenesis (Duncan *et al.*, *Dev. Biol.* 154:1-10 (1992); Ellmeier *et al.*, *Dev. Dyn.* 203:163-173 (1995); Jen *et al.*, *Dev. Dyn.* 208:92-106 (1997)). In general, Id1, Id2 and Id3 are expressed in dividing neuroblasts of the central nervous system (CNS) up to about embryonic day (E) 12.5, after which Id2 expression persists in neurons that are undergoing maturation in both the future cerebellum and cerebral cortex. Id1 and Id3, but not Id2, are expressed in endothelial cells in the brain, whereas Id1, Id2 and Id3 are expressed in endothelial cells throughout the rest of the embryo during development (Jen *et al.*, *Dev. Dyn.* 208:92-106 (1997), and Lyden *et al.*, *Nature* 401:670-677 (1999)). In adult animals, Id1 and Id3 are no longer expressed in the brain, but Id2 expression remains in the Purkinje cells of the cerebellum (Neuman *et al.*, *Dev. Biol.* 160:186-195 (1993); and Lyden *et al.*, *Nature* 401:6670-677 (1999)).

It also is known that angiogenesis, the branching and sprouting of capillaries from pre-existing blood vessels, occurs in the yolk sac and in the embryo, particularly in the brain (for a review see, for example, Risau *et al.*, *Nature* 386:671-674 (1997)).

Signaling, from both VEGF and Tie-2 receptors has been implicated in this process, as well as in tumor angiogenesis. However, little is known of the involvement of bHLH proteins during, these processes.

In the initial stages of angiogenesis, data suggests that endothelial cells are recruited to tumor sites from neighboring blood vessels (Ogata *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 9219-9222 (1993), Holash, D. & Folkman, J. *Cell* 86, 353-64 (1996), Yankopoulos, G.D. *et al.* *Nature* 407 242-8 (2000); Folkman, J. *et al.* *Nature* 339,58-61(1989)) and/or from circulating endothelial precursor cells (CEPs). Raffi, S, *J. Clin. Invest* 105 17-19 (2000); Asahara, T. *et al.*, *Science*, 275, 964-7 (1997); Kalka, C, *et al.* *Oric, Nat'l Acad Sci USA* 97 3422-7 (2000); Takahashi, T. *et al.*, *Nat Med* B5, 434-8 (1999); Peichev, M. *et al.* *Blood* 95 952-8 (2000 Feb. 1)). Several studies have shown that genetically marked bone marrow (BM) cells, when transplanted into the recipient animals can be mobilized to the peripheral circulation, but the phenotype and functional role of these cells in the regulation of angiogenesis has not previously been established.

In view of the devastating effect that can result from neuronal and cell proliferative disorders, there is a need in the art to provide therapeutic methods that can treat, ameliorate, or prevent these disorders through drug design strategies that enable *in vitro* or *in vivo* investigation of agents that affect the activity and/or expression of Id gene products in various physiological settings. There is also a need for models which serve to elucidate the functional role of bHLH proteins in cell proliferative disorders. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The present invention provides novel knockout mammals having a disruption in at least one and at most three alleles of inhibitor of differentiation genes, Id1 and Id3, wherein the disruption results in reduction or prevention of a cell proliferative disorder in the mammal as compared to a wild-type mammal. The knockout mammal can be further genetically transformed with a construct that is capable of producing a cell proliferative disorder spontaneously in the mammal. The construct preferably contains an oncogene or a proto-oncogene. Alternatively, a cell proliferative disorder is induced into the mammal via tumor xenografts.

According to a preferred embodiment of the invention, the knockout mammal is heterozygous for Id 1 gene and homozygous for Id3 gene. In one preferred embodiment, the knockout mammal is homozygous for Id1 gene and heterozygous for Id3 gene; most preferably the knockout mammal is Id1^{-/-}, Id3^{+/-}. Id disruption may affect transcription, translation, and/or post-translational modification of a polynucleotide encoding at least one gene product of Id1, Id3, or both.

According to another preferred embodiment of the invention, the cell proliferative disorder comprises cancer induced by tumor xenografts or by genetic transformation in wild-type or knockout mammals. Cancer preferably comprises breast cancer, lung cancer, lymphoma, or a combination thereof. Knockout mammals exhibit long-term survival rate against these cancers partly because of their inability to vascularize or metastasize tumor cells.

According to another aspect of the invention, there is provided an *in vivo* or an *in vitro* system for screening, and evaluating drugs useful in the treatment or prevention of the neuronal and cell proliferative disorders, including tumor vascularization, vasculature mimicry and angiogenesis.

In another aspect, the invention is directed to a method of preventing, ameliorating, or treating a cell proliferative disorder, a neurogenic disorder, or both in a subject in need thereof, comprising administering to the subject a physiologically effective amount of an agent capable of interaction with expression and/or activity of at least one inhibitor of differentiation (Id) gene product in the body of the subject. The subject can be a human subject. Preferably, the agent has an agonistic or an antagonistic affect on expression and/or activity of one or more Id gene products, more preferably the agent antagonizes expression and/or production of one of Id1 or Id3 gene products, most preferably the agent antagonizes activity and/or expression of Id1 gene products.

Additionally, the agent of the invention is administered to an individual suffering from a neurological, cell proliferative disorder, or both through the use of cell or gene therapy techniques. These techniques include, for example, introducing a cell population, preferably the individual's own cells, to the individual, wherein cells have been transformed *in vitro* with a polynucleotide molecule encoding and expressing in the body of the individual a biologically effective amount of an antagonist of one or more gene products of Id1, Id3, or both. Preferably, the antagonist is tetracycline.

According to a preferred embodiment of the invention, Id agonists or antagonists are administered to patients suffering from cancer characterized by inappropriate Id gene products activity and/or expression, along with one or more standard anti-cancer drugs, including cytotoxic or chemotherapeutic agents.

5 Another aspect of the invention features a method to screen agents for use in treating neurological and/or cell proliferative disorders. The screening test is performed *in vitro* or *in vivo*. In an *in vivo* drug screening test, the agent to be tested is administered to a mammal, and the level of expression and or activity of at least one gene product of Id1, Id3, or both is determined in the presence and absence of the test agent. The agent is
10 selected on the basis of its interaction with expression and/or activity of at least one gene product of Id1, Id3, or both, as compared to control, for use in treating said neurological and/or cell proliferative disorders. In an *in vitro* drug screening method, mammalian cells, as opposed to mammals, are employed, and mammalian cells are incubated in the presence and absence of a test agent. The level of expression and/or activity of at least one gene
15 product of Id 1, Id3, or both is determined in the presence and absence of the test agent. An agent that interacts with mammalian cell expression and/or activity of at least one gene product of Id1, Id3, or both, is selected for use in treating neurological and/or cell proliferative disorders.

 In another aspect, the invention is directed to a diagnostic method of
20 determining whether a subject has, or is at risk for, developing a neurological and/or an angiogenic disorder, comprising the steps of: a) obtaining a sample from the subject; b) determining level of expression and/or activity of at least one gene product of Id1, Id3, or both, in the subject; and b) detecting presence or absence of a genetic mutation in the subject, wherein the genetic mutation results in inappropriate or aberrant one or more Id
25 product activity and/or expression. The genetic mutation identifies a subject that has or is at risk for developing a neurogenic or cell proliferative disorder or disease. Detection of gene mutation is determined using diagnostic test kits which employ probes that specifically hybridize to one or more Id gene or Id gene products. The probes are nucleic acid molecules, or peptides, for example an antibody.

30 These and other such objects will readily be apparent to one of ordinary skill in the art.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. Tumor growth in wild-type and Id knockout mice.

A-C Wild-type (C57BU6/SV129, C57BU6, SV129) and Id knockout mice (Id1+/-Id3-/-, Id1+/-Id3+/+ in the mixed genetic background) were injected intradermally with 2×10^7 B6RV2, B-CA breast carcinoma or Lewis Lung carcinoma cell lines as indicated. Tumor surface area was measured every 2 days, and the mean and standard deviations displayed for each group. The number of animals in each group is given in parentheses.

FIGURE 2. Vascularization of tumors and metastatic lesions in C57BU6/SV 129 wild-type and Id1+/-Id3-/- mutant mice.

Figure 2A (a-d): Macroscopic views of intradermal B6RV2 and LLC tumors. B6RV2 tumors grown in C57BLJ6/SV 129 Id1+/+ Id3+/+ (Fig. 2A a) and Id1+/-Id3-/- (Fig. 2A b) mice are shown in their entirety 6 days post-inoculation. Cross-sectional view of a LLC tumor recovered from wild type C57BLJ6/SV129 (Id1+/+Id3+/+) (Fig. 2A c) and mutant (Id1+/-Id3-/-) (Fig. 2A d) on day 40 post-injection demonstrates extensive necrosis and hemorrhage in the mutant.

Figure 2B (a-d): PECANVCD31 immunostained sections from day 8 control and mutant intradermal B6RV2 tumors reveal numerous blood vessels in the wild-type (16 ± 3.2 blood vessels per 200x field) (Fig. 2B a) compared to the mutant (2.7 ± 1.4 blood vessels per 200x frame) (Fig. 2B b). Blood vessels with normal appearing lumens and branches are evident in the control (Fig. 2B c). Stunted vessels with occluded lumens are present in the tumors grown in the Id1+/-Id3-/- mice (Fig. 2B d).

Figure 2C (a-e): Hematoxylin-eosin stained sections are shown for control and wild-type intradermal LLC tumor and lung metastasis. On day 20 post-injection, viable cells and normal appearing blood vessels are seen for the wild-type tumor (Fig. 2C a), in contrast to the appearance of widespread necrosis and hemorrhage in the mutant (Fig. 2C b). Widespread LLC lung metastases are seen in the control by day 20 post-injection (Fig. 2C c) with no visible microscopic tumor in all mutant lungs by day 35 (Fig. 2C d, Table 1). An example of one of the few solitary lung nodules found in the Id1 +/-Id3+/+ mice is shown in Fig. 2C e, which is relatively small, well-circumscribed and avascular.

FIGURE 3. Angiogenesis in wild-type and mutant mice following intracorneal injection of 136RV2 lymphoma and LLC cells. Mice of the indicated genotypes were photographed on day 21 post injection with B6RV2 and on day 8 for LLC. Note the extensive blood vessel network formed around the tumors derived from both cell types in the wild-type animals (A and C). In contrast, decreased tumor growth (arrows) and a complete absence of a vascular network is noted in the Id1+/-Id3-/- mutant inoculated with the B6RV2 lymphoma cells (B, arrows). Some growth of the LLC (arrows) appears in the Id mutant with hemorrhages observed (D).

FIGURE 4 α v-Integrin and MMP2 staining in vessels of wild-type and mutant B6RV2 xenografts.

Figure 4A (a-f): CD3 1/PECAM, α v-integrin and MMP2 (Fig. 4A-a, c, e) were visualized by antibody staining to adjacent tumor sections from wild-type mice sacrificed on day 8. Stunted blood vessels observed in serially-sectioned day 8 tumors from Id1+/-Id3-/- mice stained positive for CD3 UPECAM but α v-integrin and MMP2/gelatinase staining are absent (Fig. 4A b, d, f).

Figure 4B (a-b): Tissues were processed for electron microscopy on day 6 post-injection. The width of the extracellular matrix of the blood vessel in the Id mutant animal (Fig. 4B b) is greatly thickened compare to the wild-type (Fig. 4B a). Tumor cells are poorly aligned to the extracellular matrix around the blood vessel in the mutant. The blood vessel in the mutant has dense endothelial cell projections into an obstructed lumen.

E= endothelial cell T-tumor cell; L=lumen of endothelial cell.

FIGURE 5. Transplantation of wild-type bone marrow rescues tumour growth and vascular channel formation in Id mutant mice.

Figure 5A: Id mutant or irradiated Id mutant mice reconstituted by Id mutant BM failed to support tumour growth.

Figure 5B (a-f): Irradiated Id mutant mice rescued with wild-type bone marrow (BM) restores tumour growth similar to either wild-type or irradiated wild-type mice transplanted with the wild-type BM. On day 10 after tumor implantation, channel formation is observed in wild-type (Fig. 5 B a,d), absent in Id mutant (Fig.5 Bb,e) and
 5 rescued in H&E stained plugs of irradiated Id mutant mice with wild-type BM (Fig.5 Bc,f),

FIGURE 6. BM-derived cells reconstitute the angiogenic defect in Id1^{+/+} Id3^{-/-} mutant mice.

10 Figure 6A(a-c). Irradiated Id3(-) mutant mice transplanted with Id3(+) wild-type BM were stained for H&E (Fig. 6A b) and PECAM/CD31 (Fig. 6A c) which correspond to Id3 gene expression in darkfield of a blood vessel in a day 14 B6RV2 tumour (Fig 6A a, arrows).

15 Figure 6B (a-f). Transplanted β -gal(-) BM into irradiated wild-type hosts failed to stain for LacZ tumours (Fig. 6B a,d) blood vessels, and BM (Fig. 6B d inset). Transplanted β -gal(+) BM stained for LacZ in nearly all vessels in D14 B6RV2 tumours observed in both irradiated Id mutant (Fig. 6B b,e) and wild-type (Fig. 6B c,f) recipients, with LacZ + cells also detected in BM cells (Fig. 6B e,f insets). vWF stains β -gal in blood vessels
 20 (Fig. 6 B b, inset).

FIGURE 7. VEGF-induced mobilization is impaired in Id mutant mice.

Mobilized peripheral blood mononuclear cells were isolated from AdVEGF₁₆₅ treated mice and identified as either VEGFR2+ or CD11b+ cells. The VEGFR2+CD11b(-) cells were
 25 mobilized early on in the wild-type mice, but were nearly undetectable in the Id mutant mice throughout the experimental period.

Figure 7A. The representative percentages of positive populations in PBMCs are shown.

30 Figure 7B. VEGF-mobilized peripheral blood of wild-type mice gave rise to late outgrowth endothelial colonies, whereas in Id mutant mice significantly less colonies were detected

Figure 7C (a-d). Transplantation of VEGF-mobilized PBMC from wild-type β -gal + mice into lethally irradiated Id mutant resulted in the engraftment of the LacZ+ cells and reconstitution of angiogenesis in Id-mutant mice. B6RV2 cells implanted for two days onto β -gal+ engrafted BM in Id mutant mice were colonized with BM-derived LacZ+ cells (Fig.7 C a,b). Immunohistochemical analysis of day 2 tumors previously stained for β -gal+ expression demonstrated incorporation of vWF+LacZ+ vessels Fig.7 C c), decorated by VEGFR1+LacZ+ mononuclear cells (Fig.7C d). Virtually all the LacZ+ vessels also expressed VEGFR1 (Fig.7 C d).

10 DETAILED DESCRIPTION OF THE INVENTION

This invention, as described herein, demonstrates for the first time that inhibitors of differentiation (Id) genes are required to maintain the timing of differentiation in mammalian development, and identifies a role for Id proteins in neurogenic and cell proliferative disorders, including cancer vascularization, vasculature mimicry and angiogenesis, which is of clinical importance.

In one embodiment of the invention, as disclosed herein, it has been demonstrated that at least one copy of the Id1 or Id3 gene is required to prevent embryonic lethality associated with premature neuronal differentiation and angiogenic defects in the brain. The premature neuronal differentiation in the Id1-Id3 double knockout mice indicates that Id1 or Id3 is required to block the precisely-timed expression and activation of positively acting bHLH proteins during mammalian development.

Without being limited to any specific mechanism underlying the invention described herein, one possible mechanism is that the premature neuronal differentiation in the Id double knockout mice is due to the inhibition of both the expression and the activity of tissue-restricted bHLH proteins by sequestration of E protein heterodimerizing partners. Angiogenesis associated with tumor growth and metastasis in adult animals is shown to be highly sensitive to Id dosage, as even partial loss of Id function results in profound defects in the neovascularization of tumors.

In a general embodiment of the present invention, knockout mammals are generated that are unable to support the growth and metastasis of various types of tumors. In a more preferred embodiment of the invention knockout mammals have a disruption in at least one and at most three alleles of inhibitor of differentiation genes, Id1 and Id3. Most preferably, knockout mammals of the invention are Id1 +/- and Id3-/. Disruption of

Id genes affects, for example, transcription, translation, and/or post-translational modification of Id genes.

According to a preferred embodiment of the invention, Id knockout mammals having the genotype of Id1^{+/+}-Id3^{-/-} or Id1^{+/+}-Id3^{+/+} are generated and shown to fall to support the growth and metastasis of several tumor xenografts. For example, three different tumors fail to grow and/or metastasize in mice lacking 3 out of 4 of the Id1,3 alleles (Id1^{-/-}, Id3^{+/+}) and any of the tumor growth present show poor vascularization and extensive necrosis.

It is shown that the residual vasculature in the tumors grown in the Id knockout mammals no longer has on its surface $\alpha v\beta 3$ -integrin or the associated MMP2 metalloproteinase, which normally causes degradation of components of the extracellular matrix. As antagonists of DNA binding factors, Id proteins are required for the expression of genes like αv -integrin or MMP2 by virtue of its ability to sequester transcriptional repressors. Alternatively, the effect of Id on expression of these genes is indirect.

Without being limited to any specific mechanism underlying the invention disclosed herein, one possible mechanism of action for the lack of tumor vascularization in Id knockout mammals of the invention is due to the proteolysis and remodeling of the extracellular matrix, and indeed, a pronounced thickening of the extracellular matrix surrounding endothelial cells in Id knockout mammals occurs.

In one embodiment of the invention, as disclosed herein, it has been demonstrated that either wild-type bone marrow (BM) or vascular endothelial growth factor (VEGF)-mobilized circulating endothelial precursor (CEP) cells can restore neoangiogenesis in Id1^{+/+}Id3^{-/-} mice. Transplanted wild-type BM- or VEGF mobilized-derived cells were detected throughout the vasculature of inoculated tumors and in vascular channels of VEGF-loaded Matrigel plugs. The VEGF-driven mobilization of CEPs was completely abolished in Id1^{+/+}Id3^{-/-} mice. In early phases of revascularization, the tumor vessels were decorated with BM derived mononuclear cells expressing VEGFR1 suggesting that these cells may participate early in post-natal angiogenesis. These studies demonstrate that mobilization of VEGF-responsive BM-derived precursor cells is sufficient for post-natal angiogenesis. These mobilized cells by themselves can functionally rescue tumor vasculature in the Id knockout mammals indicating that mature endothelial cells are probably not required for this process.

Furthermore, it was demonstrated that in the initial phases of neoangiogenesis there is infiltration of LacZ+VEGFR1+BM derived mononuclear cells around developing vessels. Since CEPs and hematopoietic precursor cells, such as myelomonocytic cells, express VEGFR1 (Sawano, A., *et al. Blood* **97**, 785-791.

(2001), it is possible that both of these cell types are mobilized to the neo-angiogenic vascular bed. These studies indicate that BM-derived hematopoietic precursor cells as well as CEPs may be required for early neo-angiogenesis. Some of the VEGFR1+ cells may incorporate into the vessel wall, undergo apoptosis, or recirculate to other neoangiogenic processes such as sites of metastasis.

These studies, utilizing the knockout mammal of the present invention, elucidate the role of BM-derived VEGF-responsive CEPs in the regulation of post-natal angiogenesis and lay the foundation for modulating Id1^{+/+}Id3^{+/+} CEPs to inhibit tumor angiogenesis or to accelerate wound healing. The model would also be useful in further studies aimed at determining whether simultaneous mobilization of hematopoietic precursor cells are essential for incorporation of CEPs.

Without being limited to any specific mechanism underlying the invention disclosed herein, one possible mechanism of action for the lack of tumor vascularization in Id knockout mammals of the invention is that disruption of Id 1 and/or Id3 may result in the interference of VEGF receptor signalling, thereby resulting in the failure of CEPs to mobilize to the peripheral circulation.

In accordance with another preferred embodiment of the invention, wild-type or knockout mammals are made to spontaneously become oncogenic or cancer prone through genetic transformation by an oncogene or a proto-oncogene. Oncogenic mammals of this invention are used, for example, to test physiological interaction between oncogenicity, and inhibitor of differentiation gene products *in vivo*. For example, an Id knockout and oncogenic mammal is tested for its reduced incidence of neoplasm development, compared to an oncogenic mammal, not having a mutation or disruption of the Id gene.

The knockout mammal of this invention has a variety of uses depending on the Id gene or genes that have been suppressed. Where the Id gene or genes suppressed encode proteins believed to be involved in neurogenesis, the mammal is used to screen for agents useful for neurodegenerative diseases, for example, agents that either enhance or inhibit the activity and/or expression of one or more Id gene product. Where the Id gene or genes suppressed encode proteins believed to be involved in a cell proliferative disorder, such as

cancer or angiogenesis, the mammal is used to screen for agents useful for treating or preventing these disorders.

Knockout oncogenic, or wild-type oncogenic mammals, according to the invention described herein, are used, for example, to test material suspected of being a carcinogen.

- 5 Such tests are performed by exposing the animal to the material and determining neoplastic growth as an indicator of carcinogenicity. This test can be extremely sensitive because of the propensity of the knockout animals to develop tumors.

- 10 The mammals, or cell line derived therefrom, are also used to control the regulation of the bHLH transcription cascade in mammals. The control is achieved, for example, by identifying downstream or upstream-acting chemical regulators of the transcription cascade on the expression of Id gene or loss or gain of expression of other genes. Such studies are achieved by using, for example, chip array analysis of RNA of the Id knockout mammals and comparing the pattern of a particular RNA species to the
15 pattern found in a normal mammal.

- This method is especially useful when constitutive expression of bHLH transcription genes that are involved in manifestation of an identifiable phenotype or genotype are deleterious to the growth or health of the mammal. This method is also useful to define distinct states of growth arrest or differentiation, and thereby providing a
20 molecular mechanism coupling growth arrest and differentiation. For example, exit from the cell cycle into a pre-differentiation state of post-mitotic growth arrest can be characterized by changes in the level of the activity or expression of Id genes. Other uses are readily apparent to one of skill in the art.

- In another embodiment of the invention, methods of screening agents useful in
25 treating neurological and/or cell proliferative disorders, are disclosed. The screening methods for suitable agents are performed in both an *in vitro* and *in vivo* settings. In a preferred embodiment of the invention, agent screening is performed *in vitro* using mammalian cell culture. In this case, mammalian cells are incubated in the presence and absence of a test agent, and the level of expression and or activity of at least one gene
30 product of an Id gene, for example, Id1, Id3, or both are determined in these cells.

Mammalian cells include, for example, normal mammalian cells, transformed mammalian cells, including those that are made to have aberrant or mutated Id, tumor cells, transformed oncogenic and Id knockout cells, or a combination thereof. An agent

that demonstrates interaction with expression and/or activity of one or more Id gene product is selected as a potential drug for treating or preventing a cell proliferative disorder, such as, for example, tumor vascularization or angiogenesis. The same strategy is applied to find compounds that would be useful in suppressing, a neurogenic disorder
5 observed in many patients, if such disorders are accompanied by an aberrant or abnormal production of an Id gene product.

According to a more preferred embodiment of the invention, agent screening is performed *in vivo* using test and control mammals. Depending on the type of agents to be screened and the desired affect, various mammals are used as test or control. For example,
10 wild-type mammals, knockout mammals, knockout and oncogenic mammals, knockout and tumor xenografts mammals, wild-type and oncogenic mammals, wild-type and tumor xenografts mammals, or a combination thereof are used in an agent screening test.

Included within the scope of this invention are methods for preventing, ameliorating, or treating a cell proliferative disorder, a neurogenic disorder, or both in
15 patients. These methods comprise administering to the patients a physiologically-effective amount of an agent capable of interaction with expression and/or activity of at least one inhibitor of differentiation (Id) gene product in the body of the patient.

According to a more preferred embodiment of the invention, interaction between the agent and one or more Id gene products is an antagonistic interaction. For example,
20 patients suffering from cancer may experience an elevated level of an Id protein. It would be desirable to identify therapeutic agents that reduce the level of Id proteins, which in turn reduce or eliminate vascularization, and/or metastasis of tumor, by administering to the patient a therapeutic agent capable of producing such effects.

In general, unwanted cell proliferation results from inappropriate Id protein
25 expression and/or activity. Id proteins are expressed in different types of cells including cancer cells, cells surrounding a cancer cell (stromal cells), endothelial and smooth muscle cells. For example, an increase in Id protein activity of endothelial cells surrounding cancer cells may lead to an increased vascularization of the tumor, thereby facilitating growth of the cancer cells by angiogenesis.

Alternatively, or as well as tumor angiogenesis, an increase in Id activity may
30 generate vasculature channels that facilitate tumor perfusion independent of tumor angiogenesis. This phenomenon, which is referred to as "vasculature mimickery" (see, Maniotis et al., Am. J Pathol. 155(3):739-52 (1999) incorporated herein by its entirety)

facilitates regeneration of vasculature channels that assist tumor perfusion in highly invasive tumors. It has been found that neither normal melanocytes nor poorly invasive melanoma cells generated these patterned channels in vitro under identical culture conditions, even after the addition of conditioned medium from metastatic pattern forming melanoma cells, soluble growth factors, or regimes of hypoxia.

Thus, inappropriate Id protein activity can contribute to a cell proliferative disorder in different ways such as, for example, through increasing the production of growth factors, causing aberrant growth of a cell, and increasing formation and spreading of blood vessels in solid tumors thereby supporting tumor growth.

Included within the scope of this invention are diagnostic methods for determining whether a subject has, or is at risk of developing, a neurological and/or an angiogenic disorder. The method comprises a) obtaining a sample from a subject b) determining level of expression and/or activity of at least one gene product of Id1, Id3, or both; in said subject; and b) detecting, presence or absence of a genetic mutation in the subject, wherein the genetic mutation comprises an alteration in the activity and/or expression of at least one gene product of Id1, Id3, or both. The presence of a genetic mutation in one or more of the Id genes or gene products identifies a subject that has, or is at risk for developing, a neurogenic or cell proliferative disorder or disease.

Based on known nucleotide sequences of human Id genes, one of ordinary skill in the art, employing the techniques for genotyping mice according to the invention disclosed herein, can easily design nucleotide primers for human Id genes and use the primers to detect a mutation in one or more Id genes of a human.

Further included within the scope of this invention are methods for reducing or inhibiting tumor vasculature in a subject through cell and/or gene therapy techniques. One such technique requires, for example, introducing a nucleic acid molecule, by a vector, on its own or as integrated in transformed cells, to an individual, wherein the nucleic acid molecule encodes or affects production of gene products that can interact with one or more Id gene products in vivo. Preferably, the antagonist is tetracycline. According to a more preferred embodiment of the invention, the transformed cells are the individual's own cells, and upon administering to the individual, stably express therapeutic Id gene products within the individual's body.

As used herein "Cell proliferative disorders" refer to disorders wherein unwanted cell proliferation of one or more subset of cells in a multicellular organism occurs

resulting in harm (e.g., discomfort or decreased life expectancy) to the multicellular organism. Cell proliferative disorders occur in different types of animals and in humans, and include cancers, blood vessel proliferative disorders, and fibrotic disorders.

As used herein "Inappropriate or aberrant Id product activity and/or expression " includes, for example, any change in the activity and/or expression of an Id product, as compared to the normal activity and/or expression of the Id product, including, for example, Id protein expression in cells which normally do not express Id protein; lack or reduction of Id protein expression in cells which normally do express Id protein; increased Id protein expression resulting, in unwanted cell proliferation or mutations leading to constitutive activation of Id protein; a change in the molecular structure of one or more Id genes, or gene products; reduction of Id protein activity and/or expression leading to excessive cell differentiation. The existence of inappropriate or aberrant Id product levels or activities is determined by procedures well known in the art.

"Id product", Id gene product", or "Id protein" is used interchangeably herein and includes any protein, peptide, polypeptide, polynucleotide, in sense or antisense orientation.

As used herein, "physiologically effective amount" refers to an amount capable of producing an "affect" on the production and/or activity of at least one Id gene product.

The term "affect" is defined broadly herein and encompasses any type of interaction, including, but not limited to antagonistic or agonistic interactions. Furthermore, agents having both antagonistic and agonistic affect on one or more Id gene products are also included within the scope of the invention.

The "agent" of this invention includes any compound, composition or small molecule that interacts with activity and/or expression of one or more inhibitor of differentiation gene product(s) *in vitro*, *ex vivo*, or *in vivo*. The agents can be, for example, any protein, peptide, polypeptide, nucleic acid molecule, including DNA, RNA, DNA/RNA hybrids or an antisense molecule, small molecules, antibiotics, and the like.

The agent, according to the invention, is used to treat a cell proliferative or a neurogenic disorder by administering a therapeutically effective amount of the agent to a patient in need thereof.

The agent also is used *in vitro* studies to investigate the mechanism of action of the Id proteins, and interaction between Id and other genes and gene products in the angiogenic cascade in various clinical setting.

As used herein, the term "knockout" includes, for example, a partial or complete
5 suppression of the expression of at least a portion of a product encoded by an endogenous DNA sequence in a cell. Preparation of a knockout mammal can be achieved by methods known in the art. For a review see, for example, Pfeffer *et al.*, *Cell* 73:457-467 (1993)) which describes mice in which the gene encoding the tumor necrosis factor receptor p55 has been suppressed. These mice showed a decreased response to tumor necrosis factor
10 signaling. Fung-Leung *et al.*, *Cell* 65:443-449 (1991); *J. Exp. Med.*, 174:1425-1429 (1991)) describe knockout mice lacking expression of the gene encoding CD8. These mice were found to have a decreased level of cytotoxic T cell response to various antigens and to certain viral pathogens such as lymphocytic choriomeningitis virus.

The knockout mammal of this invention is made, for example, by introducing a
15 nucleic acid construct that suppresses expression of an Id1 or Id3 gene into an undifferentiated cell type, such as embryonic stem cell. This cell is then injected into a mammalian embryo, where it then is integrated into the developing embryo. The embryo is then implanted into a foster mother for the duration of gestation.

Knockout mammals are typically produced by introduction of a knockout
20 construct into the genome of the mammal. "Knockout constructs" encompass nucleic acid sequences that are designed to decrease or suppress expression of a protein encoded by endogenous DNA sequences in a cell. The nucleic acid sequence used as the knockout construct is typically comprised of (1) DNA from some portion of the Id gene (exon sequence, intron sequence, and/or promoter sequence) to be suppressed and (2) a marker
25 sequence used to detect the presence of the knockout construct in the cell. The knockout construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to prevent or interrupt transcription of the native DNA sequence. Such insertion usually occurs by homologous recombination (i.e., regions of the knockout construct that are homologous to endogenous DNA sequences hybridize to each other
30 when the knockout construct is inserted into the cell and recombine so that the knockout construct is incorporated into the corresponding position of the endogenous DNA). The knockout construct nucleic acid sequence may comprise 1) a full or partial sequence of

one or more exons and/or introns of the Id gene to be suppressed, 2) a full or partial promoter sequence of the Id gene to be suppressed, or 3) combinations thereof.

As used herein "disruption of the gene" and "gene disruption" include, for example, insertion of an exogenous nucleic acid sequence into one region of the native
5 DNA sequence (usually one or more exons) and/or the promoter region of a gene so as to decrease or prevent expression of that gene in the cell as compared to the wild-type or naturally occurring sequence of the gene. When a construct containing this exogenous nucleic acid sequence is transfected into a cell, the construct integrates into the genomic DNA.

10 The term "progeny" refers to any and all future generations derived and descending from a particular mammal, i. e., a mammal containing a knockout construct inserted into its genomic DNA. Thus, progeny of any successive generation are included herein such that the progeny, the F1, F2, F3, generations and so on (indefinitely) are included in this definition.

15 Included within the scope of this invention is a mammal in which two or more genes have been knocked out. Such mammals can be generated by repeating the procedures set forth herein for generating each knockout construct, or by breeding mammals, each with a single gene knocked out, and screening for those with the double or single knockout genotypes. This procedure is defined as "intercrossing", herein.

20 The DNA sequences to be used to knock out a selected gene are obtained using methods well known in the art such as those described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, ColdSpring Harbor, N. Y (1989)). Such methods include, for example, screening a genomic library with a cDNA probe encoding at least a portion of the same gene in order to obtain at least
25 a portion of the genomic sequence. Alternatively, if a cDNA sequence is to be used in a knockout construct, the cDNA is obtained by screening, a cDNA library with oligonucleotide probes or antibodies (where the library is cloned into an expression vector). If a promoter sequence is to be used in the knockout construct, synthetic DNA probes are designed for screening a genomic library containing the promoter sequence.

30 Another method for obtaining the DNA to be used in the knockout construct is to manufacture the DNA sequence synthetically, using a DNA synthesizer.

The DNA sequence encoding the knockout construct must be generated in sufficient quantity for genetic manipulation and insertion into embryonic stem (ES) cells.

Amplification is conducted by 1) placing the sequence into a suitable vector and transforming bacterial or other cells that can rapidly amplify the vector, 2) by PCR amplification, or 3) by synthesis with a DNA synthesizer.

This invention further contemplates production of knockout mammals from any species of rodent, including without limitation, rabbits, rats, hamsters, and mice. Preferred rodents include members of the Muridae family, including rats and mice. Generally, the embryonic stem cells (ES cells) used to produce the knockout mammal will be of the same species as the knockout mammal to be Generated. Thus, for example, mouse embryonic stem cells will usually be used for Generation of knockout mice.

Embryonic stem cells are typically selected for their ability to integrate into and become part of the germ line of a developing embryo so as to create germ line transmission of the knockout construct. Thus, any ES cell line that is believed to have this capability is suitable for use herein. One mouse strain that is typically used for production of ES cells, is the 129J strain. The cells are cultured and prepared for DNA insertion using methods well known to the skilled artisan such as those set forth by Robertson in: *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. IRL Press, Washington, D.C. (1987); Bradley *et al.*, *Current Topics in Devel. Biol.*, 20:357-371 (1986); and Hogan *et al.*, *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (1986).

Insertion of the knockout construct into the ES cells is accomplished using a variety of methods well known in the art, including for example, electroporation, microinjection, and calcium phosphate transformation.

For insertion of the DNA sequence, the knockout construct DNA is added to the ES cells under appropriate conditions for the insertion method chosen. Where more than one construct is to be introduced into the ES cell, DNA encoding each construct can be introduced simultaneously or one at a time.

Screening is done using a variety of methods. When the marker gene is an antibiotic resistance gene, the cells are cultured in the presence of an otherwise lethal concentration of antibiotic. Those cells that survive have presumably integrated the knockout construct. If the marker gene is other than an antibiotic resistance gene, a Southern blot of the ES cell genomic DNA is probed with a sequence of DNA designed to hybridize only to the marker sequence. Finally, if the marker gene is a gene that encodes

an enzyme whose activity can be detected (*e.g.*, beta-galactosidase), the enzyme substrate is added to the cells under suitable conditions, and the enzymatic activity is analyzed.

The knockout construct is integrated into several locations in the ES cell genome, and integrates into a different location in each cell's genome, due to the occurrence of random insertion events. The desired location of the insertion is in a complementary position to the DNA sequence of Id1 or Id3 genes. Typically, less than about 1-5 percent of the ES cells that take up the knockout construct actually integrate the knockout construct in the desired location. To identify those cells with proper integration of the knockout construct, the DNA is extracted from the cells using standard methods such as those described by Sambrook *et al.*, *supra*. The DNA is then probed on a Southern blot with a probe or probes designed to hybridize in a specific pattern to genomic DNA digested with (a) particular restriction enzyme(s). Alternatively, or additionally, the genomic DNA is amplified by PCR with probes specifically designed to amplify DNA fragments of a particular size and sequence (*i.e.*, only those cells containing the knockout construct in the proper position generate DNA fragments of the proper size).

After suitable ES cells containing the knockout construct in the proper location have been identified, the cells are inserted into an embryo. The suitable stage of development for the embryo is very species dependent. However, for mice it is about 3.5 days. The embryos are obtained, for example, by perfusing the uterus of pregnant females. Suitable methods for accomplishing this are known to the skilled artisan, and are set forth by Bradley *et al.*, *supra*.

After ES cells have been introduced into the embryo, the embryo is implanted into the uterus of a pseudopregnant foster mother. While any foster mother maybe used, they are typically selected for their ability to breed and reproduce well, and for their ability to care for their young. Such foster mothers are typically prepared by mating with vasectomized males of the same species. The stage of the pseudopregnant foster mother is important for successful implantation, and it is species dependent. For mice, this stage is about 2-3 days pseudopregnant.

Offspring that are born to the foster mother are screened for the presence of the knockout construct using Southern blots and/or PCR as previously described. Intercrossing is achieved, for example, by crossing offspring that possess the construct to each other, if they are believed to carry the knockout construct in their germ line, to generate homozygous knockout animals. If it is unclear whether the offspring will have germ line

transmission, they are crossed with a parental or other strain and the offspring screened for heterozygosity. The heterozygotes are identified by Southern blots and/or PCR amplification of the DNA, as set forth above.

5 The heterozygotes then are crossed with each other to generate homozygous knockout offspring. Homozygotes are identified by Southern blotting of equivalent amounts of genomic DNA from mice that are the product of this cross, as well as mice that are known heterozygotes and wild type mice. Probes to screen the Southern blots are designed as set forth hereinabove.

10 Other means of identifying and characterizing the knockout offspring are available. For example, Northern blots are used to probe the mRNA for the presence or absence of transcripts encoding either the Id1 or Id3 genes, the marker gene, or both. In addition, Western blots are used to assess the level of expression of the Id1 or Id3 genes in various tissues of these offspring by probing the Western blot with an antibody against Id I or Id3 protein encoded by the gene knocked out, or an antibody against the marker
15 gene product, where this gene is expressed. Finally, in situ analysis (such as fixing the cells and labeling with antibody) and/or FACS (fluorescence activated cell sorting) analysis of various cells from the offspring are conducted using suitable antibodies to look for the presence or absence of the knockout construct Id gene product.

As used herein, an "oncogene" or "proto-oncogene", includes, for example, a
20 polynucleotide which, when incorporated into the genome of the animal, increases the probability of the development of neoplasm (particularly malignant tumors) in the animal. There are several means by which an oncogene is introduced into an animal embryo (See, for example, U.S. Patent No. 4,736,866, incorporated herein by its entirety). One such method is to transfect the embryo using a vector containing an already translocated
25 oncogene. Other methods involve modifying the oncogene or its control sequences prior to introduction into the embryo. Other methods use an oncogene whose transcription is under the control of a synthetic or viral activating promoter, or to use an oncogene activated by one or more base pair substitutions, deletions or additions.

According to a preferred embodiment of the invention, oncogene sequences are
30 introduced at the fertilized oocyte state to ensure that the oncogene sequence will be present in all of the germ cells and somatic cells of the knockout animal. Introduction of the oncogene sequence at a later embryonic stage might result in the oncogene's absence from some somatic cells of the founder animal, but the descendants of such an animal that

inherit the gene will carry the activated oncogene in all of their germ cells and somatic cells.

An oncogene, or proto-oncogene encompasses any foreign sequences or any homologous endogenous sequences, and includes for example, K-ras, Ha-ras, and c-myc.

5 Oncogenes can be placed under the regulatory control of, for example, Mouse Mammary Tumor Virus (MMTV) or Rous Sarcoma Virus (RSV) viral promoter sequences, or the like.

The invention will be more fully understood by reference to the following examples. These examples are not to be construed in any way as limiting the scope of this
10 invention. All literature cited herein is specifically incorporated by reference.

EXAMPLES

Example 1. Id1-/-Id3-/- Mice.

15 Mice lacking one to four alleles of Id1 and Id3 are generated by intercrossing Id1+/- Id3+/-mice. Offspring lacking one to three alleles in any combination are indistinguishable from the wild type, but no animals lacking all four Id alleles are born. To determine when Id1-/-Id3-/- mice die, Id1-/-Id3+/- mice are intercrossed and the embryos examined from E8.5 to birth. Between E8.5 and E 10.5, Id 1-/-Id3+/-, Id1-/-Id3+/- and Id
20 1-/-Id3-/- embryos are represented in a 1:2:1 mendelian ratio. Id1-/-Id3-/- embryos are grossly normal up to E10.5 but reduced in size by 30% at E11.5 and E12.5. By E12.5, the mutants exhibit cranial hemorrhage and no embryos survive beyond E13.5, indicating that expression of either Id1 or Id3 is essential for viability.

The ganglionic eminences of Id1-/-Id3-/- embryos develop cavitational lesions.
25 Areas of hypocellularity form at E11.5, and by E12.5 coalesced into a cavity. Aberrant capillaries flank the cavity, which rupture at E13.5, resulting in hemorrhage throughout the ventricular system .

Example 2. Id Inhibits Neuronal Differentiation

30 Mice having Id1-/-Id3-/- mutation are examined to determine the rate of growth and differentiation of their brain cells. It is found that Id1-/-Id3-/- mutant mice have smaller brain size than the wild-type mice. The decrease in the brain size of the mutant mice may be due to a decrease in proliferation of neuroblasts, or an increase in apoptosis.

At E10.5, no significant differences in apoptosis are observed between wild-type and Id1-/Id3-/- embryos. Similarly, at E10.5, no difference is found when cell proliferation was measured by immunodetection of Ki-67, a nuclear antigen expressed in all proliferating cells except those in G0 or early G1. In E11.5 mutants, however, there are fewer
 5 proliferating cells in the neuroepithelium of the telencephalon and the rhombencephalon.

The withdrawal of neuroblasts from the cell cycle is accompanied by altered expression of cell cycle regulatory proteins. In Id1-null embryonic fibroblasts, p16, the cyclin D/cdk4 inhibitor, is upregulated. Consistent with this observation, p16 and p27 are upregulated in the mutant neopallial cortex at E11.5. The expression patterns of post-
 10 mitotic differentiation markers, microtubule-associated protein 2 (MAP2) and unique B-tubulin (UBT), are complementary to that of Ki-67

Example 3. Regulation of Neuronal bHLH Expression By Id.

To define molecular mechanism of premature neuronal differentiation in the
 15 mutants, the expression pattern of neuronal-specific bHLH genes is examined. Like myogenic bHLH proteins, a hierarchy of activities is found in the neuronal bHLH family within the determination genes (*MATH1*, *MATH3*, *MASH-1*, *Ngn1* and *Ngn2*) including the expression of differentiation effectors (*NeuroD1*, *NeuroD2* and *MATH2*) (Risau *et al.*, *Nature* 386: 671-674, (1997)).

Neuro D1 expression is similar in wild-type and mutant embryos at E9.5 and E10.5,
 20 but is increased in the ganglionic eminences at E 11.5. No differences are observed in *NeuroD2* and *NeuroD3* expression. In E 11.5 mutants, *MATH1* expression is more extensive in the rhombencephalon. *MATH2* is enhanced and more extensive in the ganglionic eminences and dorsal rhombencephalon, and *MATH3* is more extensive in the
 25 dorsal telencephalon and ganglionic eminences. By E 12.5, no differences are detectable for these markers. In the ventral telencephalon, the areas of premature *MATH3* expression are more restricted than those of *MATH2* and *NeuroD1*.

No changes in the expression of *BF-1* (Xuan *et al.*, *Neuron* 14:1141-1152 (1995)), *Dlx-2* (Price *et al.*, *Nature* 351:748-751 (1991)), *Emx-2* (Simeone *et al.*, *EMBO J.*
 30 12:2735-2747 (1993)), *Pax-6* (Walther *et al.*, *Development* 113:1435-1449 (1991)) and *Shh* (Shimamura *et al.*, *Development* 121:3923-3933 (1995)) are observed, indicating that premature neurogenesis in the mutants is not the result of alterations in telencephalic patterning. These observations, the overlapping expression of *Id1* and *Id3* in the

neuroepithelium, and the lack of a neuronal phenotype in Id1 or Id3 single knockout mice indicate that either Id1 or Id3 is required for normal neurogenesis.

Example 4. Identification of Vascular Defect in the Brain

5 Aberrant endothelial cells observed in the ganglionic eminences of Id1-/-Id3-/- embryos express CD31 normally. They form enlarged, dilated blood vessels at E11.5 and an anastomotic network at E12.5. Laminin and fibronectin are expressed normally in the basement membrane. By laminin immunostaining, an absence of branching and sprouting of capillaries into the neuroectoderm of the ganglionic eminences is detected,
10 demonstrating abnormal angiogenesis in the mutants. This is consistent with expression of Id1 and Id3 in normal vasculature in the CNS during development. Although Id1, Id2 and Id3 are expressed in blood vessels outside the CNS, Id2 expression is absent from blood vessels in the CNS.

At E12.5, reduced expression of vascular endothelial growth factor (VEGF), Flk-1
15 (VEGF receptor 2) and smooth muscle action is first noted within the vascular malformation, but not in blood vessels outside the ganglionic eminences in the mutants. Vasculogenesis is normal in the mutants. The formation of the major vessels, intersomitic vessels, perineuronal vascular plexus and endocardium is unimpaired, and yolk sac blood islands of E8.5 mutant embryos are normal.

20 The cavitation lesions in Id1-/-Id3-/- mice resemble the defect in α v-integrin-null mice. However, no differences in the expression of Id1 or Id3 are observed in α v integrin-null mice. In addition, differences in the phenotypes of these mice are noted. Capillary sprouts are present in the neuroectoderm of α v-integrin null mice unlike Id mutants, indicating that the angiogenic defect in Id1-/-Id3-/- mice may not be an obligate
25 consequence of hypocellularity. Also, α v-integrin null embryos display only dilated, not malformed blood vessels.

Example 5. Reduced Id Dosage Inhibits Growth

In order to determine if partial loss of Id function alters the vascularization and
30 growth of tumors, Id1+/- Id3 -/- mice and wild-type littermates are challenged with intradermal injections of various tumor cell lines. Wild-type Mice (129Sv/C57BL/6) inoculated with the B6RV2 lymphoma cell line show a rapid increase in tumor mass which result in death at 24.1 +/- 7.1 days when averaged over 20 animals (Figure 1A, Table 1). At

the time of death, all wild-type animals show evidence of metastasis in the mesenteric lymph nodes by gross inspection (Table 1). These results are independent of the genetic background of the wild-type mice as pure 129Sv or C57BL/6 mice as well as mixed Sv129/C57BL/6 mice all show a similar response to the tumor cell implants (Figure 1A).

- 5 A similar result is observed when wild-type mice are inoculated with a murine breast cancer cell line (B-CA), although this line displays a lower metastatic potential (Figure 1B, Table 1).

- In striking contrast, the Id1+/- Id3-1- mice are completely resistant to the growth and metastasis of the B6RV2 Lymphoma cells and the B-CA breast cancer cells. At 6 and
10 8 days post inoculation there is a small peak in growth of the B6RV2 and B-CA cells, respectively (Figure 1 A,B). However, by 10 and 20 days post-injection the small masses have completely regressed and no tumor reappears for the duration of the experiment (Figure 1 A,B). All 22 mice inoculated with B6RV2 cells remain healthy for 540 days after which time they are sacrificed and examined for evidence of lymph node
15 involvement. None of the animals shows any histological evidence of metastasis in mesenteric lymph nodes (Table 1).

Table 1. Outcome of Tumor Cell Implantation Experiments

Tumor Cell Type	Genotype	Overall Survival (total animals)	Survival Duration (days)	Metastases (total animals)
¹ B6RV2	Id1+/-Id3-/-	(22/22)	⁴ 540	(0/22) M
	Id1+/-Id3+/+	(15/15)	⁴ 540	(0/15) M
	Id1+/+Id3+/+	(0/20)	24.1±7.1	(20/20) G [28.1±8.2]
² B-CA	Id1+/-Id3-/-	(15/15)	⁴ 330	(0/15) M
	Id1+/-Id3+/+	(0/10)	⁴ 60	(0/10) M
	Id1+/+Id3+/+	(0/16)	⁴ 60	(2/16) G [1]
³ LLC	Id1+/-Id3-/-	(0/20)	40.2±2.3	(0/20) M
	Id1+/-Id3+/+	(0/15)	34.2±3.7	(3/15) M
	Id1+/+Id3+/+	(0/57)	18.9±3.6	(57/57) G [7.9±4.2]

1 = Mesenteric lymph node metastases

2 = Breast Cancer cells

3 = Lung metastases

4 = sacrificed

G = gross and M=microscopic disease

[] = total mets/anim.

10

All 15 mice, inoculated with the B-CA line also remain healthy for the duration of the experiment (330 days) again with no evidence of metastasis (Table 1)

When the dosage of Id protein is increased in mice, an intermediate phenotype with respect to tumor resistance is observed. Id1+/- Id3-/+ mice inoculated with lymphoma cells show more robust tumor growth at 6 days post inoculation relative to the Id1+/-Id3-/- mice and a 4-6 day delay in the time to total regression (Figure 1A). In addition, unlike the Id1+/-Id3-/- mice, the Id1+/- Id3+/+ mice support the eventual growth of the B-CA cell line although the rate of tumor growth is significantly delayed relative to the wild-type controls (Figure 1B).

A third tumor cell line, Lewis lung carcinoma (LLC), is used to challenge the Id knockout mice in the xenograft assay. Unlike the B6RV2 or B-CA cells, the LLC cells continue to proliferate in all strains of mice, albeit at a significantly lower rate in the Id1+/- Id3-/- and Id1+/- Id3+/+ mice relative to the wild-type littermates (Figure 1c). As in the case of B6RV2, the growth rate of the LLC in wild type mice is the same in pure 129Sv and C57BL or mixed genetic backgrounds. The survival time of the Id1+/-Id3-/- mice supplanted with LLC cells is greater than twice that of the wild-type mice (with the Id1+/-Id3+/+ mice showing an intermediate phenotype (Table 1). This difference in survival time may be accounted for by the absence of metastatic lesions observed in the Id knockout mice relative to the controls (Table 1) and/or differences in the histology of the tumors.

Example 6. Angiogenic Defects in Tumors

Animals implanted with the B6RV2 lymphoma cells and LLC cells are sacrificed after 6 and 20 days, respectively and the site of injection examined by gross morphology and histology (Figure 2A-D). Wild-type animals inoculated with B6RV2 show clear evidence of tumor cell growth and blood vessel infiltration after 6 days. In the Id1+/-Id3-/- animals however, after 6 days while there appears to be some growth of the tumor cells, little if any vascularization of the mass is observed. A cross section of a primary LLC tumor grown in the wild-type animals for 20 days showed a normal appearing cellular mass with atypical tumor vascular bed. LLC cells grown in the Id1+/- Id3-/- animals, however, consistently produce a primary mass composed primarily of necrotic tissue and hemorrhage.

A typical histological section of an 8 day B6RV2 cell tumor grown in the wild-type background stained with CD3 I/PECAM shows clear evidence of normal appearing capillaries with wide lumens and branching. In the Id1+/-Id3-/- animals however, after 6

days while there appears to be some growth of the tumor cells, little, if any, vascularization of mass is observed. These tumors contain a reduced number of blood cells, and these vessels appear stunted or occluded relative to the controls. Similarly, histological analysis of sections from LLC tumors confirm the appearance of normal blood vessels throughout the tumor grown in wild-type animals. In tumors grown in the Id1+/- Id3-/- mice however, few normal blood vessels are observed with evidence of widespread hemorrhage and necrosis. Viable tumor cells are seen only at the peripheral margins.

Example 7. Tumor Metastasis

The mutant mice Id1+/-Id3-/- and wild-type mice are implanted with LLC and examined for the evidence of tumor metastasis. A striking difference is observed between the wild-type and Id mutant mice when metastasis to the lungs is examined. 51/57 of the wild-type animals show evidence of lung metastasis by gross inspection at the time of death (avg. of 7.9 + 4.2 nodules) whereas 0/20 of the Id1+/- Id3-/- mice show such lesions (Table 1). In addition, none of the Id1+/- 104animals shows evidence of Microscopic disease in the lung histologically whereas metastatic lesions with blood vessel infiltration in the wild-type control animals are readily apparent. Once again, the Id1+/-Id3+/+ animals show an intermediate phenotype with 3/15 animals possessing microscopic lesions in the lung. Interestingly, these lesions are well encapsulated and show no blood vessel infiltration. Although the Id knockout animals inoculated with LLC cells eventually died by day 40 on average (see Table 1), the explanation for this remain unclear. The results suggest that the cause of death is not related to the widespread metastatic lung disease observed in the wild-type mice.

Example 8. Mode of Id Action in Tumor Angiogenesis

In order to determine if the failure of the LLC cells to metastasize in the Id knockout animals is solely due to a failure of the cells in the primary lesion to enter the bloodstream (perhaps due to the defect in the vascular bed observed), LLC cells are injected into the tail veins of wild-type and Id1+/- Id3-/- mice and the appearance of metastases to the lungs quantitated. The wild-type animals display extensive metastatic disease in the lung with blood vessel infiltration after 8 days (6/6 animals tested). In sharp contrast, in the Id1+/- Id3-/- group, 5/6 animals show no evidence of lung metastasis after 21 days with the remaining animal showing an avascular, well encapsulated tumor nodule.

From these analyses, it is concluded that the failure of LLC cells to metastasize to the lungs is not only due to a failure of the cells in the primary tumor to transmigrate through the endothelial cells, but rather the homing and/or establishment of the tumor cells in the lungs is also defective in these animals.

5 To rule out the possibility that the failure in tumor growth in the Id knockout animals is due to an anti-tumor immune response, tumor cells are implanted into the corneal layers of the eye which are avascular and therefore have no inherent cell mediated immunity. Both the B6RV2 line (after 21 days) and the LLC cells (after 8 days) show extensive growth in the wild-type animals (Figure 3A). In both cases, extensive growth of blood vessels into the tumor mass is observed. In sharp contrast, when this experiment is performed on the Id1+/- Id3-/- mice, minimal tumor growth of the B6RV2 is observed after 21 days with little or no vascularization of the tumor mass (Figure 3B; 6 animals tested). Similarly, the LLC cells grow to a minimal size in the Id1+/- Id3-/- mice after 8 days with no clear vascular network development (Figure 3D; 6 animals tested). Some hemorrhage is observed in the region of the tumor mass in these animals.

The results show that there is no cellular immune infiltration in the tumors or tumor remnants in the Id knockout animals and the growth of the tumors is independent of the genetic background (see Figures 1-4). In addition, ablation of NK cells with an anti-asialo antibody in the Id1, 3KO animals fails to restore the ability of the lymphoma cell line to grow or metastasize.

Example 9. Association Between MMP2, $\alpha v\beta 3$ -Integrin and Angiogenesis

The levels of αv -integrin and MMP2 on the endothelial cells of the tumors grown in the wild-type and Id knockout mice are determined in order to find whether the association of soluble MMP2 metalloproteinase with $\alpha v\beta 3$ -integrin is required for angiogenesis during tumor development. B6RV2 lymphoma cells are grown in a wild-type host containing normal appearing blood vessels that stained positively with an anti-CD31 (PECAM) antiserum (Figure 4A, panel 1; 9 animals tested). This staining co-localizes with staining for αv -integrin (Figure 4A, panel c. When the B6RV2 cells are grown in the Id1+/-Id3-/- host, most of the anti-CD31 positive cells enclose no lumen at all but occasionally blood vessels are observed with very narrow lumens (Figure 4A panel b; 9 animals tested). Importantly, the CD31 positive cells enclosing the lumen are completely negative for αv -integrin (Figure 4A, panel d). Since $\alpha v\beta 3$ -integrin has been shown to

recruit soluble MMP2 metalloproteinase, the expression of MMP2 on the surface of the tumor vasculature should also be affected. MMP2 staining is readily detected on the endothelial cells within tumors grown in a wild-type host (Figure 4A, panel e) but is absent on the endothelial cells in the Id1+/- Id3-/- animals (Figure 4A, panel f).

5 The deficiency of endothelial cells of the tumors grown in the Id knockout mice in metalloproteinase activity, is shown to be related to a thickening of the extracellular matrix around the forming blood vessels. Electron microscopic analysis of the endothelial cells present in the B6RV2 tumors grown in wild-type and Id1+/- Id3-/- mice show that a typical capillary within the B6RV2 tumor mass grown in wild-type animals endothelial
10 cells (E) adjacent to a relatively narrow layer of ECM (Figure 4b, panel a, between the arrows). In the Id1+/- Id3-/- animals however, the lumens of the capillaries are obstructed by what appeared to be cytoplasmic projections consistent with the gross histological data presented above (Figure 4A, panel b). In addition, the ECM adjacent to the endothelial cells shows a marked thickening relative to that observed in the control animals. Taken
15 together, these observations are consistent with the possibility that one defect in the Id1+/-Id3-/- endothelial cells is a failure to display active MMP2 on the surface of the endothelial cells resulting in an inability of the newly forming blood vessels to form a functional vascular network. This ultimately results in the regression of the B6RV2 tumor mass.

20

Example 10 Wild-type bone marrow cells and restoration of angiogenesis

Mutant Id +/-Id3-/- mice were lethally irradiated, reconstituted with donor wild-type bone marrow (BM) cells and the inoculated intradermally with B6RV2 lymphoma cells (Fig. 5a). In these reconstituted mice, tumor growth paralleled that observed in wild-
25 type animals. Moreover, as in the wild-type animals, Id mutant mice engrafted with wild-type BM developed widespread mesenteric lymph node metastases and died prior to day26. In contrast, rapid tumor regression was observed in non-transplanted Id mutant mice or irradiated Id mutant mice receiving Id mutant BM cells.

To determine whether the contribution of wild type donor BM in Id mutant mid5
30 was restricted to tumor vasculature, vascular channel formation in VEGF loaded Matrigel plugs implanted subcutaneously in the abdominal cavity of the host animal. (Fig. 5B) Ten days after plug implantation, histological analysis showed no vascular channel formation in Id mutants (n=10) (Fig. 5B, b,e) while widespread vessel sprouting can be seen in the

VEGF-loaded Matrigel plugs of the wild-type mice (Fig. 5B a,d). Notably, reconstitution of the Id mutant mice (n=12) with wild-type BM cells completely restored vascular channel formation. These data demonstrate that the wild-type BM cells are sufficient for the restoration of tumor growth and vascular channel formation in Id mutant host animals.

5

Example 11. Bone Marrow Derived Cell Recruitment and Angiogenesis

To assess whether the BM-derived cells were recruited to the neo-angiogenic site in B6RV2 tumors, RNA *in situ* hybridization for Id3 was performed (Fig. 6). Id3 expression was detected in tumor associated endothelial cells in irradiated Id1+/-Id3-/- mice (n=12) transplanted with donor Id1+/+Id3+/+ wild type BM (Fig. 6A a,b) Platelet endothelial cell adhesion marker (PECAM/CD31) co-expression with Id3+ cells established the presence of mature blood vessels (Fig. 6A,c) To further confirm the contribution of BM-derived endothelial cells to the tumor vasculature, B6RV2 tumors were implanted into lethally irradiated Id mutant mice previously repopulated with BM derived from β -galactosidase (β -gal+) knock in mice (Rosa 26). Since Rosa 26 mice express β -gal transgene in all tissues, LacZ staining of the tumor tissue can reveal donor derived BM cells readily. In tumors implanted for 14 days, a homogenous distribution of LacZ+ vessels was detected throughout the tumor tissue (Eosin stained) (Fig. 6 B b,e) Greater than 95% of the vessels expressing von Willebrand Factor (cWF) were characterized as Lac+ (Fig. 2b, inset). These results demonstrate that wild-type BM derived cells are incorporated extensively into vessels associated with B6RV2 tumors grown in Id mutant mice.

The contribution of donor bone marrow cells to the tumor vasculature may have reflected the inability of the neighboring Id1 and/or Id3 deficient endothelial cells to be recruited to the neo-angiogenic vascular bed, thereby forcing the recruitment of donor BM cells. To examine the relative contribution of pre-existing and BM-derived CEPs in a more physiologically relevant model, BM from β -gal+ Rosa 26 mice was transplanted into lethally irradiated wild-type mice (n=12) and then challenged with tumor. As in tumor vessels of BM reconstituted Id mutant mice, LacZ stained blood vessels were detected throughout the vasculature of the B6RV2 tumor grafts in the wild type host animal (Fig. 6B c,f) In addition, LacZ staining was detected in all BM cells verifying a complete engraftment of the host BM, whereas the bone itself showed only eosinophilic staining (Fig. 6B e,f Insets) No LacZ staining was seen in either the BM or tumor tissue

30

when BM cells of the wild type, β -gal(-) mice were transplanted (n=8) (Fig. 6B a,d Insets). Collectively, these results underscore the capacity of BM-derived cells to be mobilized to the tumor vascular bed and to contribute to the neo-angiogenic process.

5 ***Example 12. Association of Angiogenesis and VEGF-induced mobilization of circulating endothelial precursor (CEP)***

One explanation for the impairment of post natal angiogenesis in Id1+/-Id3-/- mutant mice is the inability of CEPs to mobilize in response to VEGF₁₆₅. To test this hypothesis, Id1+/-Id3-/- mutant and wild-type mice were injected with adenoviral vectors
10 carrying VEGF₁₆₅ transgene (AdVEGF₁₆₅) which allowed for the release of VEGF (average plasma level of 750 pg/ml) into the peripheral blood circulation at levels comparable to that observed in the presence of tumors. Elevation of plasma VEGF₁₆₅ levels in the wild type mice (n=6) induced mobilization to the peripheral circulation of a large percentage of mononuclear cells with the CEP potential expressing vascular
15 endothelial growth factor receptor -2) (VEGFR2, Flk-1) that lacked the myelomonocytic marker (DC11b) (11% on day 3, 5% on day 5 and .2% on day 10) (Fig 7A) in Id mutant mice (n=6). The VEGFR2+ cells were most likely BM derived CEPs rather than mature endothelial cells since they were able to form VEGFR2 late outgrowth colonies of endothelial cells (CFU-EC) I *in vitro* cultures (Fig. 7B). These data support the notion that
20 defective angiogenesis observed in Id1+/-Id3-/- mutant mice is a consequence of impaired VEGF-induced mobilization of CEPs.

The sustained release of VEGF in mice also induces mobilization of hematopoietic stem cells. To determine if transplantation of VEGF mobilized cells can rescue hematopoiesis and reconstitute angiogenesis in lethally irradiated Id mutant mice, β Gal +
25 Rosa 26 mice were injected with AdVEGF and mobilized cells harvested from the peripheral circulation were transplanted into lethally irradiated Id mutant mice. Similar to reconstitution with BM-derived cells, transplantation of VEGF-mobilized cells resulted in restart of angiogenesis and tumor growth of the implanted B6RV2 in Id mutant mice. LacZ stained cells can be seen in the blood vessels of the B6RV2 tumors (Fig 7C a,b),
30 demonstrating that VEGF-mobilized angio-competent CEPs are required for the induction of angiogenesis. Immunohistochemical analysis of day 2 tumors demonstrated the presence of vWF+LacZ+ vessels (Fig. 7C,c) decorated by VEGFR1+Lac+ mononuclear cells (Fig. 7Cd). In addition, virtually all the LacZ+ vessels also expressed VEGFR1 (Fig.

7Cd) These data suggest that in the early phases of neo-angiogenesis recruitment of VEGF responsive BM-derived cells, composed of CEPs and VEGFR1+ precursor cells with morphologic features reminiscent of hematopoietic cells may be required to initiate the neo-angiogenic process.

5

Example 13. Genotyping by PCR

Genomic DNA is obtained from mouse tail tips and yolk sacs as described by Hogan *et al.*, *J. Embryol. Exp. Morphol.* 97:95-110 (1998) incorporated herein by its entirety. PCR analysis is performed with primers specific for the wild-type and targeted alleles. Primer sequences for Id1 are pr-22 (common oligonucleotide; 5'-CCTCAGCGACACAA GATGCGATCG-3'), pr-k4 (wild-type oligonucleotide; 5'-GGTTGCTTTTGAACGTTCTGAACC-3') and pr-pgk (mutant oligonucleotide; 5'-GCACGAGACTAGTGAGACGTG3'). Primer sequences for Id3 are yz 151 (common oligonucleotide; 5'-GTTTTGAACATAGGTCTGCC-3'), yz 170 (wild-type oligonucleotide; 5'- CACCGGGCTCAGCGCCTTCAT-3'), and yz 29 (mutant oligonucleotide; 5'- TCGCAGCGCATCGCCTTCTA-3'). PCR cycling conditions are 90°C for 30 s, 57°C for 30s and 65°C for 3 min, for 40 cycles. The amplified PCR products are analyzed on 1 % agarose gels to separate the wild-type (1.0 kb for Id1 and 2.0 kb for Id3) and targeted allele (0.8 kb for Id1 and 2.5 kb for Id3) fragments. PCR genotyping of α v-integrin-null mice is performed as described by Bader *et al.*, *Cell* 95:507-519 (1998) incorporated herein by its entirety.

20

Example 14. Morphological and Histological Analysis

Embryos are obtained from timed pregnancies, with noon of the plug date defined as E0.5. The plug date is the date that embryos are removed. Embryos are fixed in 4% paraformaldehyde. Paraffin embedding is performed by dehydrating embryos through ethanol and HistoClear (National Diagnostics) before immersion in paraplast (Fisher Scientific). Sections of 6 or 7 micrometer are stained with hematoxylin and eosin (H&E).

25

Tumors, lung and enucleated eyes are fixed, processed and stained with H&E as described above. The entire lung per animal is sectioned and analyzed for metastases by two independent scorers. Blood vessels are counted in eight random 200x fields and results from two independent scorers are averaged.

30

Example 15. *In situ* Hybridization

Embryos and tumors are fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6 or 7 micrometer) are processed for *in situ* hybridization with (alpha-³³P)UTP-labeled antisense RNA probes, as described by Manova *et al.*, *Dev. Dyn.* 213: 293-308 (1998) incorporated herein in its entirety. Probe templates are provided by R. Kagqama (MATH-1, MATH-2 and MATH-3) Akazawa *et al.*, *J. Biol. Chem.* 270:8730-8738 (1995), S. Tapscott (NeuroD2, NeuroD3) McCormick *et al.*, *Mol. Cell. Bio.* 16:5792-5800 (1996), J. Lee (NeuroD) Lee *et al.*, *Science* 268:836-844 (1995); E. Lai (BF-1)20, E. Boncinelli (Emx2) Simonson *et al.*, *Nucleic Acids Res.* 21: 5767-5774 (1993), P. Gruss (Pax6) Walther *et al.*, *Development* 113:1435-1449 (1991), J. Rubenstein (Dlx2) Price *et al.*, *Nature* 351:748-751 (1991), and A. McMahon (Shh) (Shimamura *et al.*, *Development* 121:3923-3933 (1995)).

Example 16. Whole-mount Immunohistochemistry

Embryos are processed as described by Winnier *et al.*, *Genes Dev.* 11, 926-940 (1997). Embryos are incubated with primary antibody (MEC 13.3 rat monoclonal anti-mouse PECAM-1 antibody; Pharmingen). Samples are incubated with biotinylated anti-rat antibody (Vector), and then with peroxidase-conjugated avidin (Vector). For colour detection, NiCl₂ and DAB are added to embryos

Example 17. Immunohistochemistry

For Ki67 immunohistochemistry, tissue antigens are unmasked and sections are incubated with monoclonal anti-mouse Ki67 antibody (NCL-Ki67-MMI; Novocastra Laboratories), followed by biotinylated anti-mouse antibody. The Histomouse-Sp Kit (Zymed Laboratories) is used. For all other antigens the following antibodies are used: MAP2 and p16, anti-rat MAP2 (clone MM-2; Sigma) or monoclonal anti-p16 (Santa Cruz) antibody; PECAM (CD31), MEC 13.3 monoclonal anti-mouse PECAM-1 antibody (Pharmingen); laminin, polyclonal anti-laminin antibody (Sigma); VEGF, and VEGF (Santa Cruz), Flk-1, and Flk-1 (Sigma); smooth muscle alpha-actin-anti-alpha-actin (Sigma); alpha-integrin antibody (Chemicon); MT2 (gelatinase-A), polyclonal anti-mouse NIMP-2 antibody (Sigma). All sections are then incubated with the appropriate biotinylated secondary antibodies (Vector).

Example 18. Cell Lines

5 B6RV2, a murine leukemia/lymphoma cell line generated at Memorial Sloan-Kettering Cancer Center and LLC, obtained from American Type Culture Collection, are used. B-CA, breast carcinoma cell line, is established from tumors generated by crossing Id1-nuD mice and mammary tumor virus-polyoma virus knockout mice. All lines are maintained in DMEM with 10% fetal calf serum.

Example 19. Tumor Implantation In a Murine Model

10 Roughly 2×10^7 cells of each tumor cell line are injected intradermally in the right lower abdomen. Surface area is measured by two independent scorers (Dial Caliper, and Scianeware). For intravenous injection of tumor, 2×10^6 LCC cells are injected in the tail veins of anesthetized mice (2.5% Avertin). For the eye implantation studies, animals are anesthetized with 2.5% Avertin and proparacaine hydrochloride ophthalmic solution
15 (0.5%). Roughly 2×10^6 B6RV2, LLC cells or media alone are injected with a Hamilton syringe and needle with the assistance of a dissecting microscope (Zeiss Olympus) into the corneal layers of the eye.

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Example 20. Electron microscopy

Tissues are processed with "yellowfix" (2.5% glutaraldehyde, 4% paraformaldehyde, 0.02% picric acid in 0.1 M Na-cacodylate). The samples are post-fixed with 1% osmium tetroxide-1.5% ferricyanide, dehydrated in ethanol and infiltrated with
25 Spurr's Resin. Tissue blocks are trimmed with a diatome diamond knife (Diatome USA) on RMC NMOO ultramicrotome. Sections are contrasted with lead citrate and viewed on a JEOL 100CX-II electron microscope.

Example 21. Preparation of Knockout Constructs

30 The DNA sequence to be used in producing the knockout construct is digested with a particular restriction enzyme selected to cut at a specific location(s) such that a new DNA sequence encoding, for example, a marker gene to be inserted in the proper position within this DNA sequence. The proper position for marker gene insertion

depends on factors such as the restriction sites in the sequence to be cut, and whether an exon sequence or a promoter sequence, or both are to be interrupted (i.e., the precise location of insertion necessary to inhibit promoter function or to inhibit synthesis of the native exon). In some cases, it is desirable to actually remove a portion or even all of one or more exons of the Id 1 or Id3 gene to be suppressed so as to keep the length of the knockout construct comparable to the original genomic sequence when the marker gene is inserted in the knockout construct. In these cases, the genomic DNA is cut with appropriate restriction endonucleases such that a fragment of the proper size is removed.

The marker gene is any nucleic acid sequence that is detectable and/or assayable. However, typically it is an antibiotic resistance gene or other gene whose expression or presence in the genome is easily detected. The marker gene is usually operably linked to its own promoter or to another strong promoter from any source that will be active or can easily be activated in the cell into which it is inserted. However, the marker gene need not have its own promoter attached, as it may be transcribed using the promoter of the gene to be suppressed. In addition, the marker gene normally has a polyA sequence attached to the 3' end of the gene. This sequence serves to terminate transcription of the gene. Preferred marker genes are any antibiotic resistance gene such as neo (the neomycin resistance gene) and beta-gal (beta-galactosidase).

After the genomic DNA sequence has been digested with the appropriate restriction enzymes, the marker gene sequence is ligated into the genomic DNA sequence using methods well known to the skilled artisan and described in Sambrook et al., supra. The ends of the DNA fragments to be ligated must be compatible. This is achieved by either cutting all fragments with enzymes that generate compatible ends, or by blunting the ends prior to ligation. Blunt ending of the sequence is achieved using methods well known in the art, such as for example by the use of Klenow fragment (DNA polymerase I) to fill in sticky ends. The ligated knockout construct is inserted directly into embryonic stem cells, or it is first placed into a suitable vector for amplification prior to insertion. Preferred vectors are those that are rapidly amplified in bacterial cells such as, viral vectors or pBluescript II SK vector (Stratagene, San Diego, Calif.) or pGEM7 (Promega Corp., Madison, Wis.).

Example 22. Bone Marrow transplantation for Tumour and Matrigel plug assays.

Mice were genotyped by polymerase chain reaction (PCR) of tail DNA as described (Lyden, D. *et al. Nature* 401, 670-7 (1999)). Id mutant (Id1^{+/+}Id3^{-/-}) and wild-type C57B1/6/Sv129 mice were lethally irradiated with 950 rads. Approximately, 1X10⁶ β -gal negative or positive (Rosa 26 mice) BM cells were injected into tail veins of irradiated recipient mice. Following four weeks, allowing for BM reconstitution, mice were injected intradermally with either 2X10⁷ B6RV2 murine lymphoma cells (established at Memorial Sloan-Kettering Cancer Center) or one ml of iced Matrigel (Becton-Dickinson) and admixed with VEGF (Peprotech, 10 μ g/ml.) and heparin (Sigma, 100 μ g/ml.) into the right lower abdomen. For the tumour, surface area was scored by three independent observers (Dial Caliper, Science Ware).

Example 23. Histological analysis, Immunohistochemistry, In situ Hybridization in Bone Marrow Recipient Mice

Tumour tissue and Matrigel plugs were fixed in 4% paraformaldehyde for four hours. Paraffin embedding was performed by dehydrating tissue and plugs through ethanol and HistoClear (National Diagnostics) before immersion in paraplast (Fisher Scientific). Sections of 8 μ m were stained with hematoxylin and eosin and antibodies to PECAM (CD31) (MEC 13.3 monoclonal antibody (mAb) anti-mouse PECAM-1 antibody (Pharmagen), vWF (combined primary and biotinylated secondary antibody (Dako), and VEGFR1 (Flt-1, biotinylated mAb, clone MF-1, ImClone Systems) were used. For *in situ* hybridization, sections were hybridized to (α -³³P) UTP labeled anti-sense RNA probes as described (Lyden, D. *et al. Nature* 401, 670-7 (1999)).

Example 24. β galactosidase (LacZ) Staining in Bone marrow Recipient Mice

Tumour tissue and femoral bones, split in two to expose marrow, were fixed in 4% paraformaldehyde for two hours. The tumour tissues were further dissected into small pieces and the marrow was flushed in whole from the bone. The samples were washed in PBS and PBS containing washing buffer solution (2mM MgCl₂ 5mM EDTA, 0.01% sodium deoxycholate, 0.02% NP-40) and stained in fresh x-gal solution at 37 °C overnight according to methods previously described¹⁶. The X-gal stained tumour and BM then were embedded in paraffin, sectioned, and counter-stained with eosin to visualize LacZ negative tissue.

Example 25. VEGF-induced mobilization

Id mutant or wild-type mice were injected intravenously with 10^5 MOI of E1-E4+ AdVEGF and as control same dosage of AdNull as previously described^{13, 17}. Mobilized peripheral blood mononuclear cells (PBMC) from AdVEGF₁₆₅ or AdNull treated mice
5 were collected by orbital bleeding and stained with FITC-conjugated anti-VEGFR2 (clone DC101) mAb and CD11b (Mac1, myeloid lineages)-Phycoerythrin. Stained cells (1×10^4) were analyzed on a Coulter Elite flow cytometer to determine the representative percentages of positive populations in PBMCs. VEGF plasma levels were measured at the time of orbital bleeding.¹³ For quantification of CEPs with early and late outgrowth
10 potential, 5×10^4 mobilized PBMCs obtained from AdVEGF₁₆₅ or AdNull treated Id mutant or wild-type mice on a day 0 to day 21 and plated in the presence of modified endothelial growth medium on collagen/fibronectin coated plastic dishes, as previously described^{11, 12, 13}. Endothelial growth medium consisted of X-vivo 20 serum free medium (BioWhittaker), supplemented with VEGF (10ng/ml), basic-FGF (5 ng/ml), heparin 10
15 units/ml, and endothelial growth supplement (Collaborative Research). Endothelial colonies (CFU-EC) were identified and quantified by co-staining with DiI-Ac-LDL metabolic labeling and vWF immunostaining^{11, 12}. Colonies that formed within the first three days (early outgrowth) and colonies that formed by 14 days (late outgrowth) were quantified by DiI-Ac-LDL labeling after the start of culture (mean \pm SEM). Transplantation
20 of VEGF mobilized PBMC from wild-type Rosa mice into lethally irradiated Id mutant were performed as described above. A total of five million VEGF mobilized PBMC from day 3 and 5 were collected, Ficoll and transplanted by tail-vein injections into lethally irradiated hosts.

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What Is Claimed Is:

1. A knockout mammal having a disruption in at least one and at most three alleles of inhibitor of differentiation (Id) genes, wherein said disruption results in prevention or reduction of a cell proliferative disorder in said mammal.
2. The knockout mammal of claim 1, wherein said knockout mammal is further genetically transformed with a construct capable of producing said cell proliferative disorder in said mammal.
3. The knockout mammal of claim 1 wherein said construct contains an oncogene or a proto-oncogen.
4. The knockout mammal of claim 1 wherein said proliferative cell disorder is induced in said mammal via a tumor xenograft.
5. The knockout mammal of claim 1, wherein said mammal is heterozygous for Id1 gene and homozygous for Id3 gene.
6. The knockout mammal of claim 1, wherein said mammal is homozygous for Id1 gene and heterozygous for Id3 gene.
7. The knockout mammal of claim 5, wherein said mammal is Id1 +/- and Id3/-.
8. The knockout mammal of claim 5, wherein said mammal is Id1+/- and Id3+/+.
9. The knockout mammal of claim 1, wherein said cell proliferative disorder is cancer.
10. The knockout mammal of claim 9, wherein said cancer comprises, breast cancer, lung cancer, lymphoma, or a combination thereof.

11. The knockout mammal of claim 9, wherein said cancer do not metastasize or vascularize in the body.
12. The knockout mammal of claim 9, wherein expression of integrin and metalloproteinase are substantially reduced in said cell proliferative disorder.
13. The knockout mammal of claim 1, wherein said mammal is a mouse.
14. The knockout mammal of claim 1, wherein said disruption affects transcription and/or translation of a polynucleotide encoding at least one gene product of Id1, Id3, or both.
15. The knockout mammal of claim I wherein said disruption affects post-translational activity of said at least one gene product of Id1, Id3, or both.
16. A method of preventing, ameliorating, or treating a cell proliferative disorder in a subject having an inappropriate or aberrant Id product, comprising administering to said subject a physiologically effective amount of an agent capable of interaction with at least one inhibitor of differentiation (Id) gene product in the body of said subject.
17. The method of claim 16 wherein said cell proliferative disorder comprises angiogenesis.
18. The method of claim 17 wherein said angiogenesis is a tumor angiogenesis.
19. The method of claim 16 wherein said interaction produces an antagonistic, or agonistic effect on expression, activity, or both of said at least one Id gene product.
20. The method of claim 16 wherein said agent comprises proteins, peptides, sense or antisense nucleic acid molecules, small molecules, or a combination thereof.

21. The method of claim 18 wherein said administration additionally comprises one or more anti-cancer agents.
22. A method to screen agents for use in treating neurological and/or cell proliferative disorders, comprising the steps of:
- a) incubating mammalian cells in the presence and absence of a test agent,
 - b) determining levels of expression and or activity of at least one gene product of Id 1, Id3, or both in said cells incubated in the presence and absence of said test agent; and; and,
 - c) selecting an agent that interacts with expression and/or activity of said at least one gene product of Id1, Id3, or both, as compared to control, for use in treating said neurological and/or cell proliferative disorders.
23. The method of claim 22 wherein said interaction is agonistic, antagonistic, or both.
24. A method to screen agents useful in treating, neurological and/or cell proliferative disorders, comprising the steps of:
- a) administering a test agent to a mammal;
 - b) determining level of expression and/or activity of at least one gene product of Id1, Id3, or both; in the presence and absence of a test agent;
 - c) selecting an agent that affects expression and/or activity of said at least one gene product of Id1, Id3, or both, as compared to control, for use in treating said neurological and/or cell proliferative disorders.
25. The method of claim 24 wherein said mammal has been altered to contain a disruption in at least one and at most three alleles of inhibitor of differentiation gene, Id1 and Id3 genes.
26. The method of claim 24 wherein said mammal is being genetically transformed with a construct that is capable of producing cancer spontaneously in said mammal.

27. The method of claim 24 wherein said mammal is implanted with one or more tumor xenograft.
28. The method of claim 27 wherein said tumor xenograft comprises xenograft of lymphoma, breast cancer, lung cancer, or a combination thereof.
29. A diagnostic method for determining whether a subject has, or is at risk for developing, a neurological and/or an angiogenic disorder comprising the steps of:
- a) obtaining a sample from said subject;
 - b) determining level of expression and/or activity of at least one gene product of Id 1, Id 3, or both; in said subject; and
 - c) detecting presence or absence of a genetic mutation in said subject, wherein said genetic mutation results in inappropriate or aberrant one or more Id product activity and/or expression, wherein said genetic mutation identifies a subject that has, or is at risk for developing, a neurogenic or cell proliferative disorder or disease.
30. A method for reducing or inhibiting, tumor vasculature in a mammal comprising: introducing a cell population to said individual, said cell population is transformed with a polynucleotide molecule encoding and expressing in the body of said individual a biologically effective amount of an antagonist of one or more gene products of Id1, Id3, or both.
31. The method of claim 30 wherein said antagonist is tetracycline.
32. A diagnostic test it for detecting the presence or absence of a genetic mutation in a subject resulting in an inappropriate or aberrant one or more Id product activity and/or expression, comprising:
- (a) a probe which specifically hybridizes to one or more Id gene, or gene products;
 - (b) a reagent means for detecting said hybridization;

wherein the probe and reagent means are each present in amounts effective to perform the hybridization assay.

33. The diagnostic test kit of claim 32, wherein said probe is an antibody.

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Fig. 1a

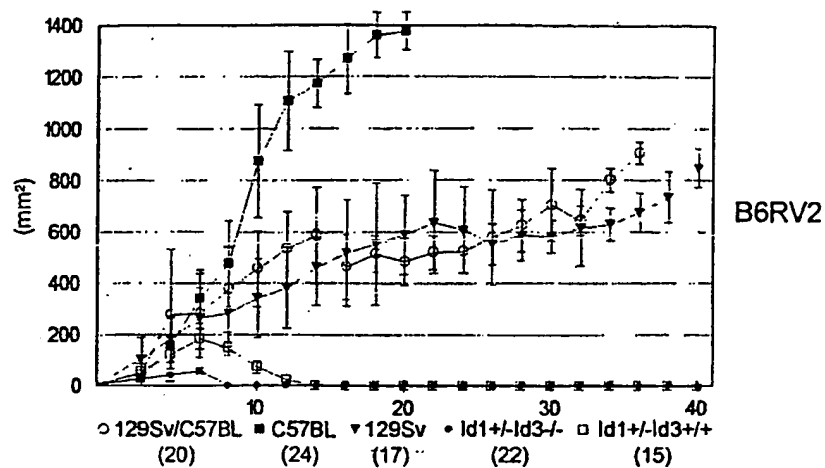


Fig. 1b

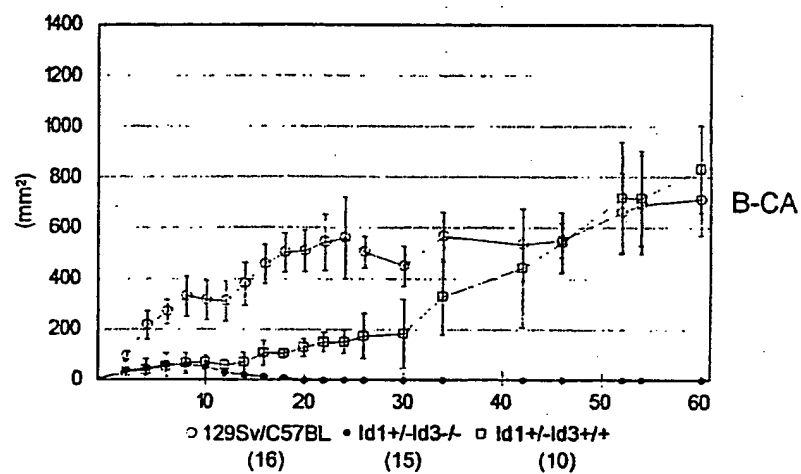


Fig. 1c

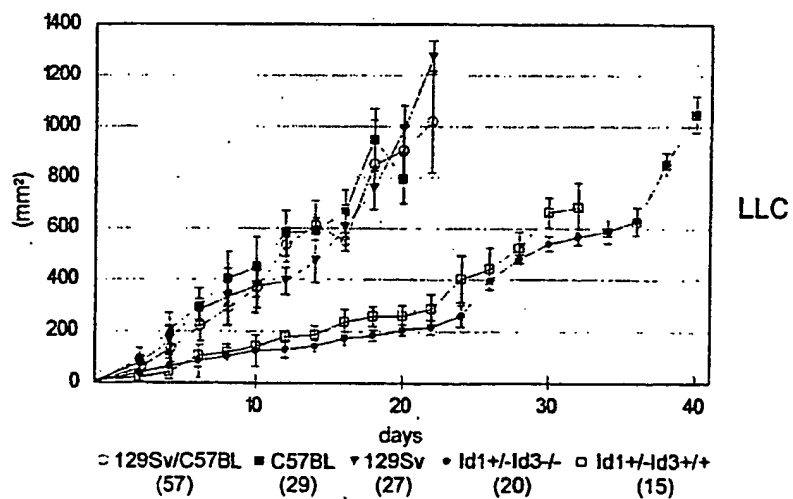


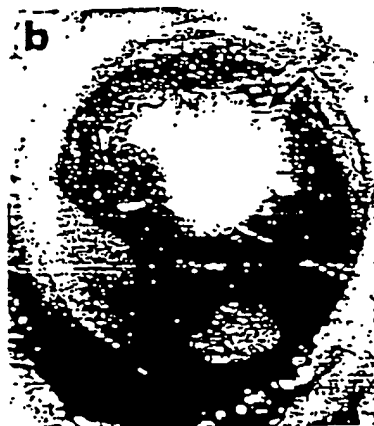
Figure 2a

Id1+/+Id3+/+



Figure 2b

Id1+/-Id3-/-



B6RV2



Figure 2c



LLC

Figure 2d

Figure 3a

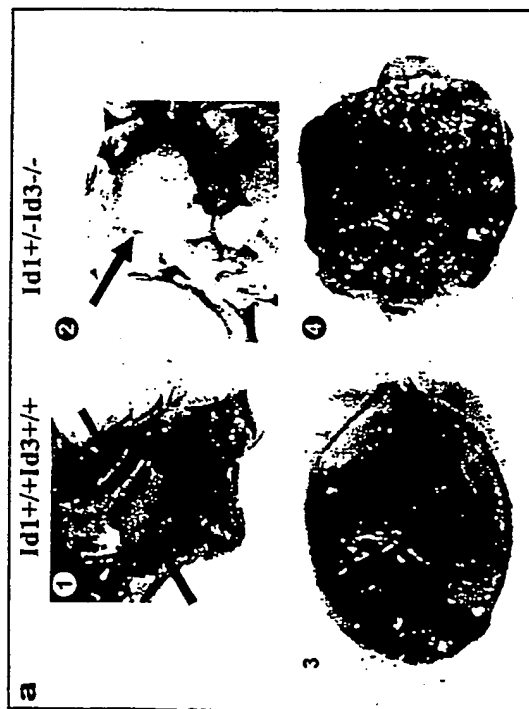


Figure 3b

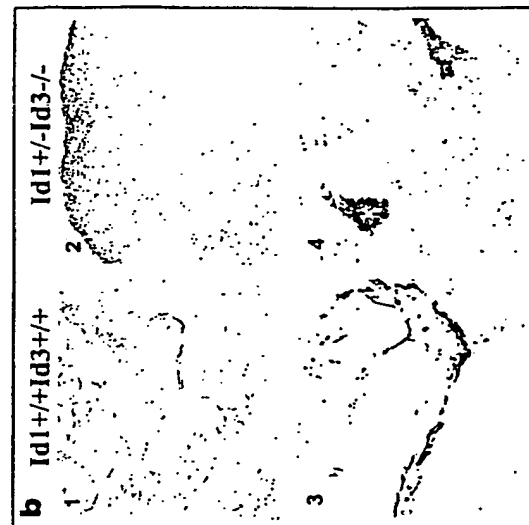
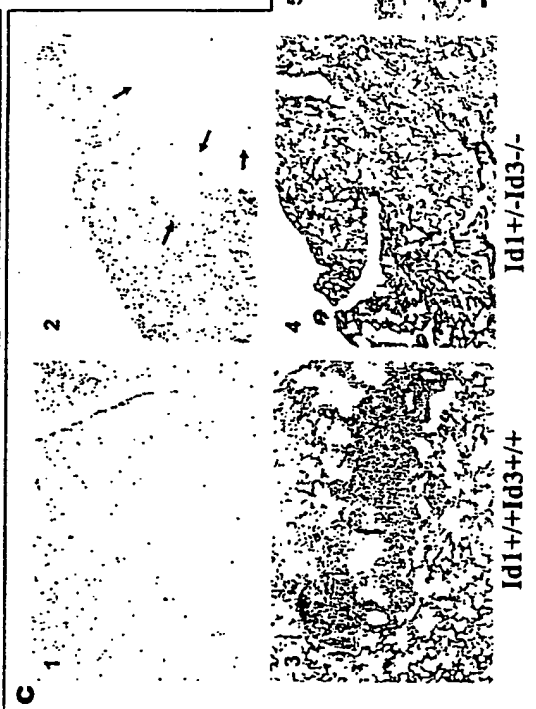


Figure 3d



Figure 3c

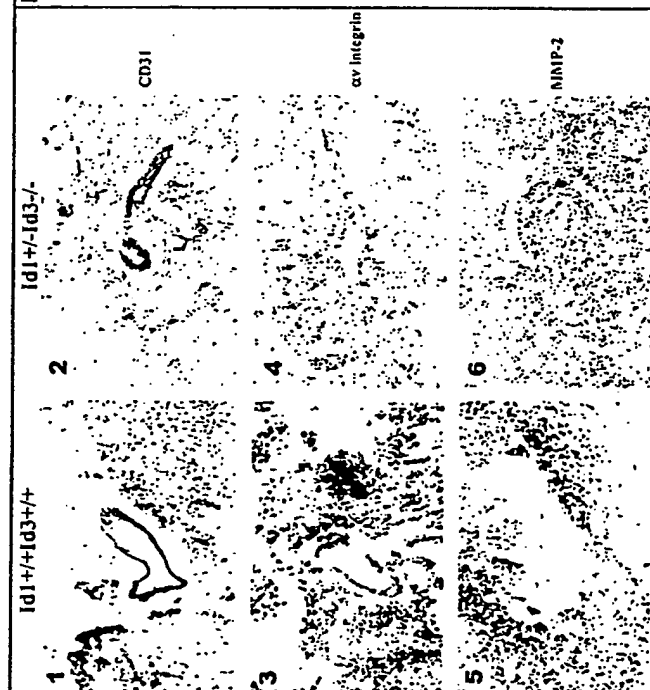


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Figure 4b



Figure 4a



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Fig. 5a

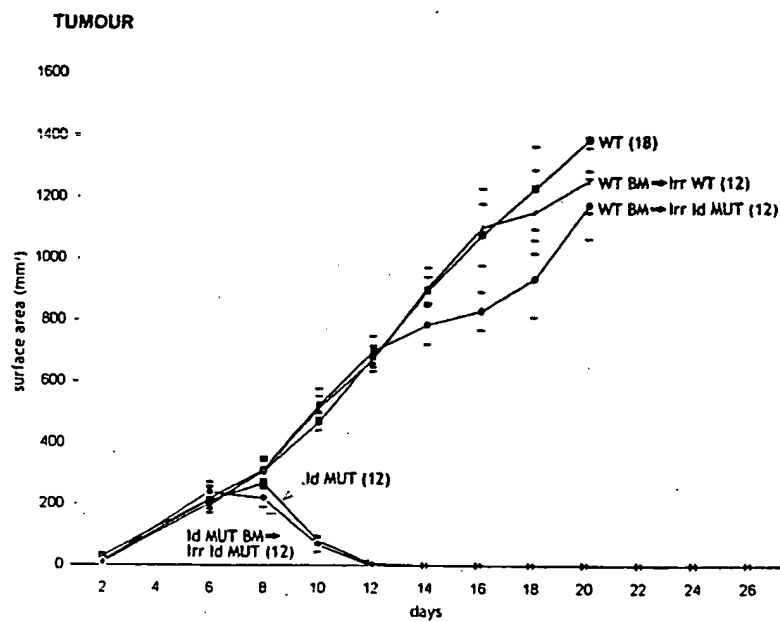


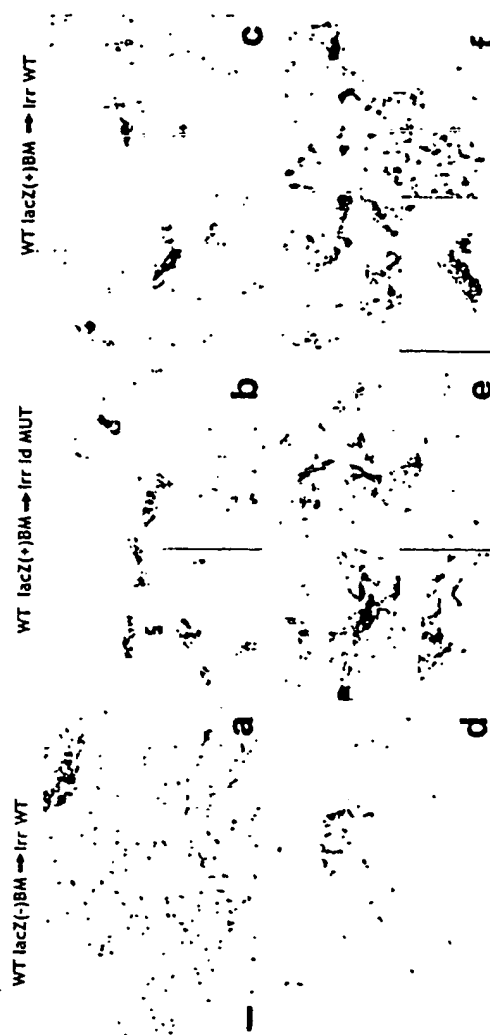
Fig. 5b

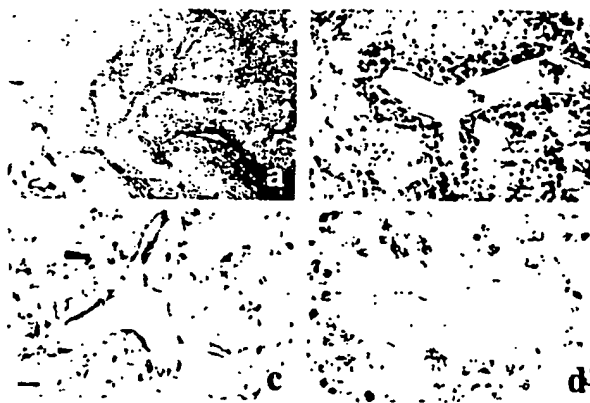
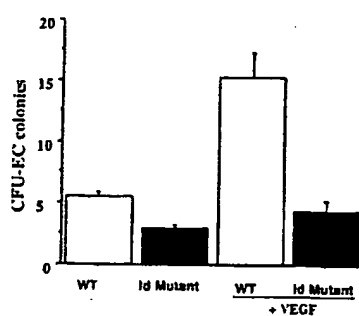
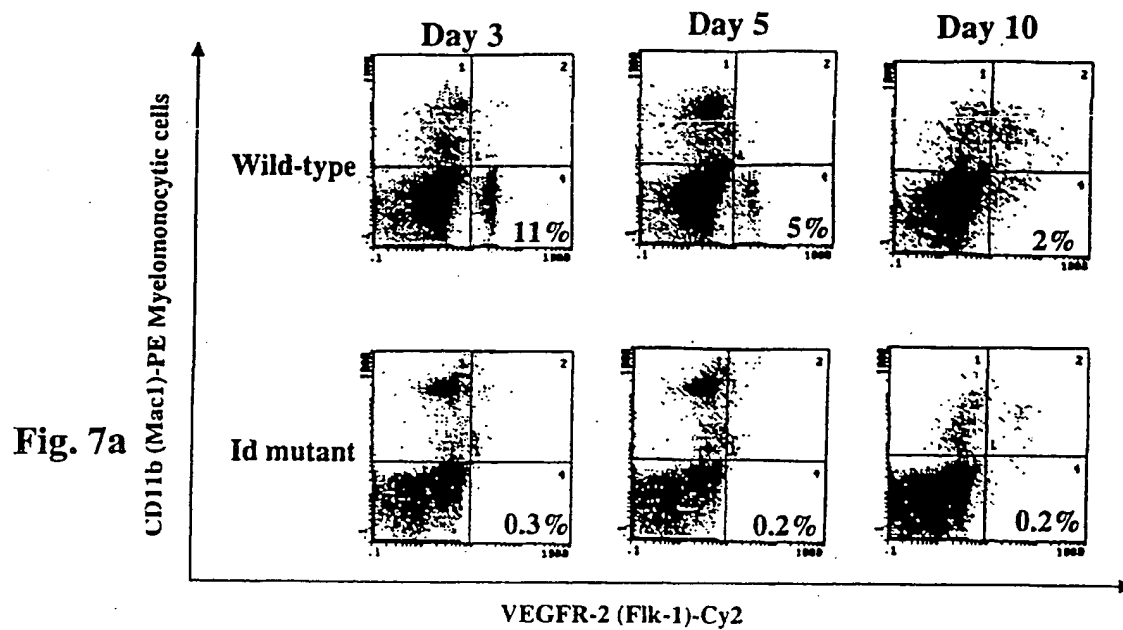


Figure 6 **A** Id3 expression



Figure 6 **B** Rgal





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/07378

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 21/00; A61K 31/00, 49/00; A01N 43/04

US CL : 800/4; 514/14; 514/44; 424/9.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/4; 514; 424/9.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, EMBASE, EAST DATA BASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YAN WEI. Genetic Analysis of the Id Genes in Mouse Development. Diss Abstr. Int. B, August 1999, Vol. 60, No. 2, page 471-B.	1-15
X,P	LYDEN, D. et al. Id1 and Id3 are Required for Neurogenesis, Angiogenesis and Vascularization for Tumor Xenografts. Nature. 14 October 1999, Vol. 401, pages 670-677. Complete article.	1-15
Y,P		16-33
Y,P	ISRALE, M. et al. Id Gene Expression as a Key Mediator of Tumor Cell Biology. Cancer Research. 01 April 1999, (7, Suppl.) Vol. 59, pages 1726-1730, entire document.	29, 32-33
A	ANDRES-BARQUIN, P. et al. Id Genes Encoding Inhibitors of Transcription are Expressed during in vitro Astrocyte Differentiaon and in Cell Lines Derived form Astrocytic tumors. Cancer Research. 15 January 1997, Vol. 57, pages 215-220.	29,32,33

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

02 AUG 2001

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/07378

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/07378

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING: This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-15 & 24-28 are drawn to a Id1 and Id3 knockout mammal and a method of screening agents for treating cell proliferative disorder.

Group II, claim(s) 16-21 are drawn to treating preventing, ameliorating or treating cell proliferative disorder by administering peptides or proteins.

Group III claim(s) 16-21 are drawn to treating preventing, ameliorating or treating cell proliferative disorder by administering sense nucleic acid molecules.

Group IV, claims 16-21 are drawn to treating preventing, ameliorating or treating cell proliferative disorder by administering antisense nucleic acid molecules.

Group V, claims 16-21 drawn to drawn to treating preventing, ameliorating or treating cell proliferative disorder by administering small molecules.

Group VI claims 22-23 drawn to an in vitro method of screening agents for treating cell proliferative disorder.

Group VII claims 29,32 and 33 drawn to a diagnostic method and a diagnostic test for presence or absence of genetic mutation.

Group VIII claims 30-31 drawn to a method for reducing tumor vasculature in a mammal by transforming cell population an antagonist.

The inventions listed as Groups I - VIII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the group I is knockout mammals with disruption in inhibitor of differentiation genes; Id1 and Id3, and method of screening agents useful in treatment of neurological and/or cell proliferative disorders by using these mammals.

The special inventive concept of group II is a method of preventive, ameliorating or treating a cell proliferative disorder in a subject by administering protein or polypeptide capable of interaction with Id gene product.

The special inventive concept of group III is a method of preventive, ameliorating or treating a cell proliferative disorder in a subject by administering sense nucleic acid capable of interaction with Id gene product.

The special inventive concept of group IV is a method of preventive, ameliorating or treating a cell proliferative disorder in a subject by administering antisense nucleic acid capable of interaction with Id gene product.

The special inventive concept of group V is a method of preventive, ameliorating or treating a cell proliferative disorder in a subject by administering small molecule capable of interaction with Id gene product.

The special inventive concept of group VI is an in vitro method of screening agents for use in treating neurological or/and cell proliferative disorder.

The special inventive concept of group VII is a method and a test for detecting genetic mutation in Id1 or/and Id3 by detecting level of gene expression.

The special inventive concept of group VIII is a method of reducing tumor vasculature in a mammal by transplanting transformed cells expressing an antagonist.

The methods of groups II-VIII do not share a special technical features: therefore each method is patentably separate.

The knockout mice of group I and the methods of groups (II-VIII) do not share a special technical features: therefore the inventions lack unity of invention and are distinct inventions.